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Laccases as effective siccatives in alkyd resins

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Abstract

This thesis is part of a project with the aim to develop an enzymatic method to cross-link unsaturated fatty acid side chains of alkyd resins. In previous project studies it was shown that a laccase mediator system (LMS) efficiently catalyzes the drying of alkyd resins and is capable to substitute heavy metal based driers in water-borne alkyd resin paints. In order to optimize the process, this work continues by studying the LMS catalyzed curing process using previously established methods such as oxygen consumption measurements, FTIR analysis and drying time recorder tests. As an additional method gas chromatography analysis was used to analyze cross-linked linseed oil, a model compound for alkyd resins. This study further addresses application specific issues, such as the storage stability of laccase and mediator in the formulated alkyd resin paint, in regards to a future application of a LMS in water-borne alkyd resin coatings at an industrial scale.

In this work, the optimal pH for the LMS catalyzed cross-linking reaction of alkyd resins was determined by means of oxygen consumption measurements at different pH conditions. The storage stability of the mediator and laccase in the alkyd resin coating was studied using oxygen consumption measurements and drying time recorder measurements. Additionally, a substrate screening was performed to investigate the specificity of the laccase mediator system towards the most common used oils in alkyd resins. Finally, a laccase mediator screening was performed to search for alternative natural laccase mediators to the synthetic mediators 2, 2‘-azinobis-(3-ethylbenzothiazoline)-6-sulphonic acid (ABTS) and 1-hydroxybenotriazole (HBT). Hereby, the natural compounds vanillin, syringaldehyde, ethyl vanillin, acetosyringone and acetovanillone were identified as laccase enhancers. Most promising drying results were gained using acetovanillone and vanillin as laccase mediators, with similar drying results to the synthetic mediator ABTS. Via gas chromatography analysis a decrease of the triglyceride corresponding peaks confirmed the cross-linking of the model compound linseed oil after treatment with a laccase mediator system. All in all, it has been clearly proven that a laccase in combination with different natural mediators catalyzes the drying of alkyd resins and has the potential to replace heavy metal based siccatives in water-borne alkyd resin paints.

Kurzfassung

Die vorliegende Diplomarbeit wurde im Rahmen eines Projektes erstellt, das zum Ziel hat, eine enzymatische Methode zur Trocknung von Alkydharzen zu entwickeln. In vorangegangenen Studien konnte bereits gezeigt werden, dass eine Quervernetzung von ungesättigten Fettsäureseitenketten in Alkydharzen mit einem Laccase-Mediatorsystem (LMS) möglich ist. Diese Methode bietet die Möglichkeit, die bisher in Alkydharzen eingesetzten toxischen Schwermetallsikkative durch ein LMS zu ersetzen.

Diese Arbeit dient dazu die bisherigen Studien fortzusetzen, um die beschriebene enzymatische Trocknungsreaktion zu optimieren. Hierfür wurden bereits etablierte Methoden verwendet, wie zum Beispiel Sauerstoffverbrauchsmessungen, FTIR Spektroskopie und Trocknungszeitrekorder-Messungen. Zusätzlich wurden in dieser Arbeit gaschromatographische Methoden zur Analyse des quervernetzen Modelsubstrates Leinöl eingesetzt. Die erhaltenen Daten zeigten deutlich eine Abnahme der Triglyceride zuzuordnenden Peaks, nach einer Behandlung von Leinöl mit einem Laccase-Mediatorsystem. Im Hinblick auf eine zukünftige industrielle Anwendung dieses biokatalytischen Verfahrens zur Trocknung von wasserbasierenden Alkydharzen wurden zudem anwendungsspezifische Untersuchungen, wie zum Beispiel Lagerungstests des Enzyms und des Mediators im Lack, durchgeführt.

Der optimale pH Wert der LMS katalysierten Trocknungsreaktion des Alkydharzes wurde in dieser Studie durch Sauerstoffverbrauchsmessungen ermittelt. Neben Trocknungszeitmessungen dienten Sauerstoffverbrauchsmessungen dazu, die Stabilität des Enzyms und des Mediators während der Lagerung im Lack zu untersuchen. Zusätzlich wurde in dieser Studie ein Substrat-Screening durchgeführt um die Spezifität des Enzyms bezüglich verschiedener Ölkomponenten im Alkydharz zu ermitteln. Mittels Mediatorscreening wurden Mediatoren gesucht, die eine umweltfreundlichere, günstigere Alternative zu den bisher verwendeten synthetischen Mediatoren 2,2'-Azino-di-(3-ethylbenzthiazolin-6-sulfonsäure) (ABTS) und 1-Hydroxybenzotriazol (HBT) darstellen. Hierbei konnten mittels Sauerstoffverbrauchsmessungen, FTIR Spektroskopie und Trocknungszeitrekorder-Messungen Vanillin, Syringaldehyd, Ethylvanillin, Acetosyringon und Acetovanillon als natürliche Laccase Mediatoren identifiziert werden. Am meisten Potential zeigten die beiden Mediatoren Acetovanillon und Vanillin mit ähnlich guten Trocknungsergebnissen wie für den synthetischen Mediator ABTS. Die erhaltenen Resultate zeigen das hohe Potential dieses biokatalytischen Trocknungsverfahrens bezüglich einer zukünftigen industriellen Anwendung in wasserbasierenden Alkydharzlacken.

Table of contents

List of figures

List of tables

1 Introduction

This work is part of a project with the aim to develop an enzymatic method to cross-link alkyd resins and establish an alternative to heavy metal based driers in water-borne alkyd resin paints. Currently, most alkyd resin paints contain cobalt-based driers despite their carcinogenic characteristics, which makes the search for alternative harmless driers a major task for the coating industry [3]. A lot of research focuses on heavy metal based driers, such as manganese or vanadium [2][3][4][5][6]. However, those heavy metal based driers still provide potential health risks [7]. Further, they might be regulated by the European Union, as it was the case for cobalt containing products in the year 2013 [8]. Since then, regulations of the European Union require that cobalt containing products need to be registered, characterized and labeled [8].

This project is a cooperation of the Austrian Center of Industrial Biotechnology (ACIB) at the Institute of Environmental Biotechnology of the Graz University of Technology and the industrial partner Allnex Austria, with the involved center located in Graz.

In previous project studies, a laccase mediator system (LMS) has been introduced as a new environmentally friendlier method for the drying of water-borne alkyd resins [1]. It was proven that a laccase-mediator system (LMS) successfully catalyzes the oxidative drying of water-borne alkyd resins, in aqueous media as well as in drying films. The main method used to monitor the LMS catalyzed oxidation reaction of unsaturated fatty acid side chains in alkyd resins was to measure the oxygen consumption. On a molecular level the cross-linking reaction was observed by FTIR analysis, which proved the decrease of double bonds due to bio-catalyzed oxidation of the unsaturated fatty acid side chains in alkyd resins. Further, an increase of the molecular weight of the alkyd resin, caused by the reaction with oxygen was observed by RAMAN. In summary, a laccase-mediator system was introduced, that has the potential to replace heavy-metal siccatives and cross-link alkyd resins. [1]

This work continues the previous studies, in order to establish a laccase mediator system as a competitive alternative to conventional cobalt-based driers. To be able to optimize the method, it is of huge importance to obtain a better understanding of the mechanisms involved in the LMS catalyzed cross-linking of alkyd resins and its influencing factors. Therefore, measurements of the oxygen consumption were performed to observe the reaction. Further, the LMS catalyzed oxidation reaction and its cross-linking products were analyzed by time-resolved FTIR spectroscopy and gas chromatography analysis. In regards to a future industrial application, issues such as the storage stability of laccase and mediator in the formulated alkyd resin paint were addressed. In addition,

various natural compounds were tested for their potential as laccase mediators, in order to determine alternative mediators to the known synthetic mediators ABTS and HBT.

The following chapters provide basic information about coatings and paints, with a main focus on alkyd resin paints. Further, the published cobalt based drying mechanism of alkyd resins is described, followed by the suggested mechanism of the laccase mediator system catalyzed drying reaction.

1.1 Coatings and paints

In the history of mankind, paints and coatings have been used long before recorded history [9]. The oldest discovered paint surfaces were found in South Africa and are estimated to be between 350,000 and 400,000 years old [9]. At an industrial scale, household-paints were produced since the industrial revolution and have been further developed over the years. In our modern world paints and coatings have become indispensable and can be found nearly everywhere covering wood, buildings, cars, household appliances, airplanes etc. Next to a decorative function, they are used to protect materials from environmental effects like UV-radiation, chemical invasion and mechanical stress and/or have a functional purposes. [9][10]

In general, the terms “paints” and “coatings” describe the material that is applied to a substrate by various application methods and form a dry adherent film on the surface. Both terms are exchangeable, with no defined difference between them. Commonly, the term “paint” is used to describe household or architectural coatings, whereas “coating” is used in a wider spectrum, especially for more sophisticated products, like for automobiles or computer components. Next to the applied material, the term “coating" refers to the dried film on the surface and the application process itself. [10]

1.1.1 Composition of coatings

Modern paints and coatings are complex mixtures of various components and although they are more sophisticated than their ancient precursors the general principle stayed the same [9]. Like their ancient precursors, modern paints consist of binder and pigments [9]. In general, the compositions of modern coatings are categorized in four groups: binders, volatile components, pigments and extenders as well as additives [10]. The formulation of coatings depends on the coated surface, the desired properties, the application method and economic or ecological restrictions [11].

Binders (or resins) are (organic) polymers, which bind together the various substances in the coating and form a film that adheres to the coated surface [10]. Different types of resins can be distinguished, like acrylics, alkyds, amino resins, epoxies, polyurethanes or silicones [10]. The oldest

forms of our modern paints are oil paints in which vegetable oils such as linseed oil or soya oil serve as binders [9]. The focus in this study lies on alkyd paints, which contain synthetic resins so called alkyd resins as binders [12].

The main components in coatings are so called **volatile components** [10]. Their purpose is to make the coating fluid enough for the application on a surface and they evaporate during and after application [10]. Until about 1945 exclusively low molecular weight organic solvents, in which the binder components dissolve, were used [10]. Emissions of those VOCs (volatile organic compounds) have become a serious concern, putting high pressure on the development of alternatives [12]. An important way to reduce emissions are water-borne coatings, where water is a major part of the volatile components [10][12]. In water-borne resins the resin is emulsified in water, unlike in solvent borne coatings, where the binder is dissolved in organic solvents [12]. Other ways to reduce the use of organic solvents is to make solvent-borne coatings highly concentrated (so called higher-solid coatings) or to not use solvents altogether, like in powder coatings or certain solventless liquid coatings [10][12].

Very important components of paints are **pigments**, which give the coating its color [10]. They are fine insoluble particles derived from grounded natural or synthetic materials, which remain suspended in the binder after film formation [10]. Inert pigments, so called **extenders** are added to increase the bulk of paints and can be used to adjust the consistency of the coating and to reduce the color of tinting pigments [9].

In order to modify certain properties of the coating small quantities of **additives** are added [10]. A large variety of additives are used in coatings, each of them providing different functions. Some examples for additives are stabilizers, flow modifiers, anti-skinning additives or catalytically active additives. These catalytic active additives include paint-drying catalysts, so called drying agents, driers or siccatives. Driers catalyze the polymerization reaction by lowering the activation energy of the cross-link formation between the binder molecules and hereby decrease the drying time of alkyd paints. [9][12]

1.2 Alkyd resin paints

Since their introduction in the 1920s, alkyd resin binders are of major importance and can be found in a broad range of coating products [13][14]. In the year 2011 alkyd resin paints were the leaders in the EU coatings market with a production of 700 000 tons per year [15].

Alkyd resins can be described as complex polyesters modified with unsaturated fatty acids. The name “alkyd” derives from the “AL” in “polyhydric ALcohols” and the “CID” in “polybasic aCIDs”, which was

modified to “KYD” [12]. They are obtained by heating fatty acids or triglyceride oils (for example linseed oil), polyalcohol (e.g. glycerol, pentaeryhritol, etc.) and polybasic acids (e.g. phthalic acid anhydride, isopthalic acid). During this reaction polyester containing fatty acids are formed. A schematic illustration of a typical alkyd resin with a linoleic fatty acid side chain is shown in figure 1. The fatty acids used for the production of alkyd resins are mainly polyunsaturated fatty acids, such as linolenic acid, a constituent in linseed oil, or linoleic acid a major constituent in sunflower oil and soya oil. [10][12]

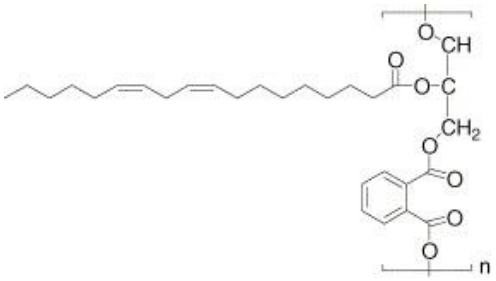


Figure 1: Schematic illustration of an alkyd resin made from phthalic acid anhydride, glycerol and linoleic acid [12].

In recent years the awareness of the environmental performance of paints has developed, taking into account not only VOC emissions but also the total ecological impact of paints. Under this approach alkyd resin paints in the form of alkyd resin emulsions, high solid alkyds or alkyd based hybrids are very interesting. First, the raw materials for the synthesis of alkyd resin paints (except for the petrochemical-based phthalic anhydride) can be gained from biologically renewable sources. Furthermore, organic solvents can be totally eliminated in alkyd resin emulsions. In addition, life-cycle assessments, which analyze amongst others the impact of the production energy, the raw materials and the greenhouse effect, showed a better performance of alkyd resin emulsions than for water-borne acrylic paints. [14]

This study focuses on the drying of water-borne alkyd paints, in which the alkyd resin is emulsified in water. Due to an increasing environmental awareness, it is expected that conventional solvent-borne alkyd paints will be replaced by environmental friendlier alkyd resin emulsions and high solid alkyds for the application on wood and metal [14]. According to a recently published report, the global market value of water-borne alkyd resins is about $52917.9 million (2012) and is estimated to grow up to $77,838.71 million by 2019 [16].

1.3 The drying of alkyd resin coatings

The drying of water-borne alkyd resin paints can be divided in two different stages [12]. The physical drying is described by the evaporation of the water [12]. During this process the interface between the binder droplets decreases and finally disappears forming a closed film [9][12]. In other words, the

oil in water emulsion inverts to a water in oil emulsion [14]. It should be mentioned that the phase inversion step happens very fast, in a matter of milliseconds, and the mechanism is not very well understood yet [17].

In the second step of the drying process, the chemical drying (also called oxidative drying) takes place, during which cross-links between the binder molecules are formed, resulting in a hardening of the film. The chemical drying is a lipid autoxidation process and involves oxidation of the unsaturated fatty acid side chains of the alkyd resins. [9][12]

1.3.1 Autoxidation of the binder

Autoxidation is as a spontaneous reaction of atmospheric oxygen with unsaturated fatty acids, whether they are present as triglycerides such as in natural oils or they are present in alkyd resin paints [18][19]. The mechanism for the autoxidation of unsaturated lipids has been an issue in many previous studies for biology, food science, cosmetics, pharmaceutics and coatings [20]. Lipid autoxidation can cause degradation of lipids and is mainly an unwanted process [20]. For example in food products it is responsible for rancidity [18]. In coatings, autoxidation is a desirable process as it is the basis of the chemical drying of alkyd resins and oil paints [20]. The autoxidation reaction of fatty acids is a radical chain reaction, terminated by the recombination of radicals and leading to a cross-linked three dimensional polymer, which protects the applied surface from environmental influences [19]. The following stages can be identified in the autoxidation reaction [9][12][21]:

1. Initiation
2. Formation of hydroperoxides
3. Hydroperoxide decomposition into free radicals
4. Polymerisation / crosslinking
5. Side reactions

(1) The reaction is initiated by a radical reaction and the abstraction of an H-atom from an unsaturated fatty acid side chain in alkyd resin binders. This reaction forms pentadienyl radicals, which react instantaneously with molecular oxygen to form peroxyl radicals. (2) Those peroxyl radicals can participate in many reactions. At the beginning of the process they mainly react to hydroperoxides by abstraction of another hydrogen atom from another substrate. Through this reaction further radicals are formed which propagate the chain reaction. (3) The formed hydroperoxides are decomposed, which can be catalyzed by cobalt-based compounds and further radicals are formed. (4) The recombination of the radicals forms peroxy, ether and carbon-carbon cross-links between the binders. Further cross-linking occurs from direct addition of radicals to double bonds. (5) Additionally to the film forming cross-linking reactions, side reactions can take

place resulting in the formation of alcohols, ketones, aldehydes, carboxylic acids, epoxides and endoperoxides.

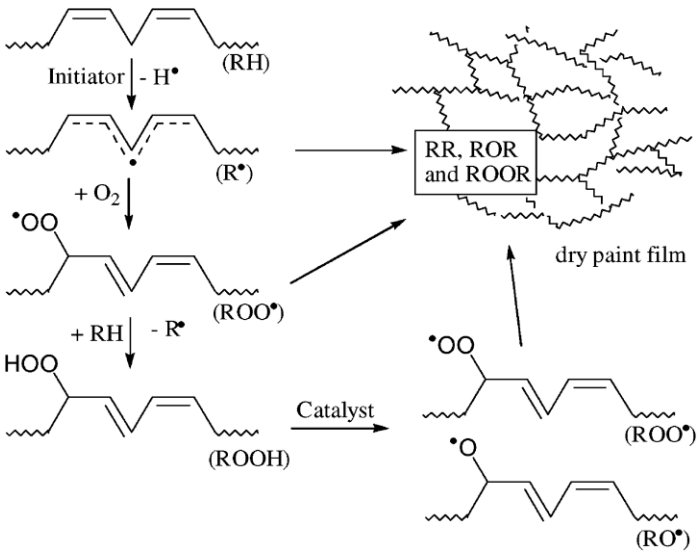


Figure 2: Proposed mechanism of the abstraction of *bis*-allylic hydrogen atoms and hydroperoxide formation in the oxidation of linoleic fatty acid side chains of alkyd resin binders [19].

Typical, unsaturated fatty acids of alkyd resins are linolenic acid (α-linolenic acid=9Z,12Z,15Z-octadecatrienoic acid and γ-linolenic acid=6Z,9Z,12Z-octadecatrienoic acid) and linoleic acid (9Z,12Z-octadecadienoic acid) [9]. These non-conjugated polyunsaturated fatty acids have a high susceptibility because of the presence of bis-allylic hydrogen atoms between the double bonds, which have a relatively low bond dissociation energy of 75 kcal/mol. In linolenic acids the presence of four bis-allylic H-atoms increases the oxidation rate compared to linoleic acids, which have only two bis-allylic H-atoms. The abstraction of monoallylic hydrogen atoms such as in oleic acids can also occur, but is less favorable since a higher dissociation energy of 88 kcal/mol is needed (figure 3). [9]

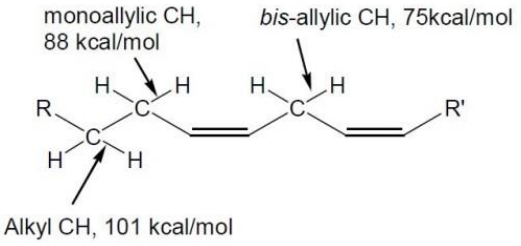


Figure 3: Different dissociation energies of the CH bonds in unsaturated fatty acids [9].

A schema of the time course of the autoxidation reaction of fatty acids in lipids is shown in figure 4. The concentration of polyunsaturated fatty acids decreases very fast at the beginning of the oxidation reaction; simultaneously hydroperoxide concentration increases and reaches a maximum. Hydroperoxide formation is then surpassed by hydroperoxide decomposition resulting in the

formation of non-volatile cross-linked products, next to numerous other oxygen containing products, such as alcohols, ketones, aldehydes and carboxylic acids. [9][12]

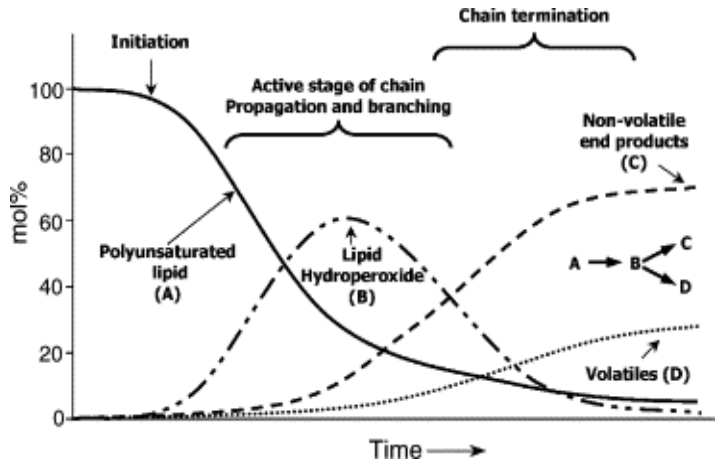


Figure 4: Time course of the autoxidation reaction of fatty acids in lipids after Gardner[22]. [12]

1.3.2 Role of cobalt compounds in the oxidation reactions of alkyd coatings

The autoxidation of the fatty acids can be catalyzed by metal ions, as it is realized in alkyd resin paints by the addition of heavy metal based driers. Without the addition of such driers the chemical drying of alkyd resins is very slow, resulting in insufficient long drying times. Metal catalyzers can catalyze the radical chain reaction through metal-dioxygen activation by direct reaction of the metal complex with the resin or by catalyzing the decomposition of hydroperoxides. Oxidation catalysts in alkyd resin paints are for example cobalt, manganese, vanadium and iron-based catalysts. [9][12][21] In most solvent-borne paints cobalt based siccatives are used as primary drier [7]. The role of the cobalt-based catalysts in the oxidation of unsaturated fatty acids is shown in figure 5 [20][21].

Oxygen absorption

$Co^{2+} + O_2 \longrightarrow [Co^{3+}OO]^{2+}$

$[Co^{3+}OO]^{2+} + RH \longrightarrow [Co^{3+}OOH]^{2+} + R\bullet$ (RH = fatty acid ester)

Hydrogen abstraction

$RH + \text{initiator} \longrightarrow R\bullet$

$Co^{3+} + RH \longrightarrow Co^{2+} + R\bullet + H^{+}$

Direct activation of the double bond

$R\text{-}CH{=}CH\text{-}R + Co^{3+} \longrightarrow R\text{-}\underset{\bullet}{C}H\text{-}CH^{+}\text{-}R + Co^{2+}$

Formation of hydroperoxides

$R\bullet + O_2 \longrightarrow ROO\bullet$

$ROO\bullet + RH \longrightarrow ROOH + R\bullet$

Decomposition of hydroperoxides

$ROOH + Co^{2+} \longrightarrow RO\bullet + Co^{3+} + OH^{-}$

$ROOH + Co^{3+} \longrightarrow ROO\bullet + Co^{2+} + H^{+}$

$2\,ROOH \longrightarrow RO\bullet + ROO\bullet + H_2O$

Figure 5: Proposed reactions as occuring in cobalt-catalyzed oxidation of alkyd resins [20][21].

1.4 Laccases

Laccases (EC 1.10.3.2, benzendiol: oxygen oxidoreductase) are blue multicopper oxidases catalyzing the oxidation of ortho- and para-diphenols, aminophenols, polyphenols, polyamines, and aromatic thiols with the concomitant reduction of molecular oxygen to water [23][24][25]. The enzyme has been first described in the 19th century as a component of latex from the Japanese laquer tree *Rhus vernicifera*, which makes it one of the oldest enzymes ever described [26]. Since then, laccases were found in every fungus examined for laccases, in few bacterial species, some insects and higher plants [26].

Laccases catalyze the removal of a hydrogen atom from the hydroxyl group of *ortho* and *para*-substituted mono- and polyphenols by a one electron abstraction under reduction of oxygen to water as it is shown in figure 6 [23][27]. During the catalytic cycle four substrate molecules are oxidized by the laccase to produce four electrons and simultaneously one molecule of oxygen is reduced to two molecules of water [27]. The formed radicals can further form dimers, oligomers or polymers [27].

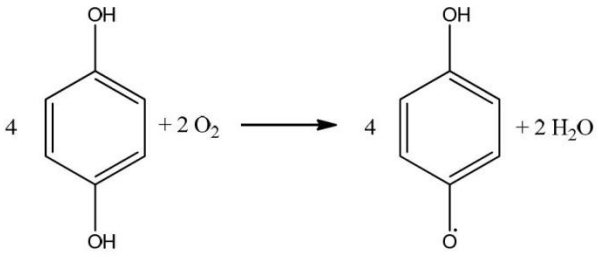


Figure 6: Laccase catalyzed oxidation of four *p*-benzendiol molecules under reduction of oxygen reacting to four benzosemiquinone molecules and two molecules of water.

The laccase molecule contains four copper atoms that are bound in one mononuclear domain containing one type 1 (T1) copper and a trinuclear cluster with one type 2 (T2) and two type 3 (T3) copper centers. The blue color of the laccases is caused by the T1 copper center, which has a characteristic absorbance around 600 nm. The T1 copper is the primary electron acceptor site, where the one electron oxidation of a substrate takes place. The electrons generated by the oxidation are transferred to the trinuclear copper site, where oxygen is reduced and water is released. In the resting state the enzyme is fully oxidized with all copper atoms in the Cu^{++} state. The fully reduced state of the enzyme is reached by the transfer of the electrons from T1 to the trinuclear copper site, which is the rate limiting step in the catalytic cycle. The reduction of molecular oxygen at the T2/T3 site is a two-step process in which oxygen bound intermediates are formed and water is released at

each step. The structure of the active site and the catalytic cycle of laccases are illustrated in figure 7 and figure 8. [27][28]

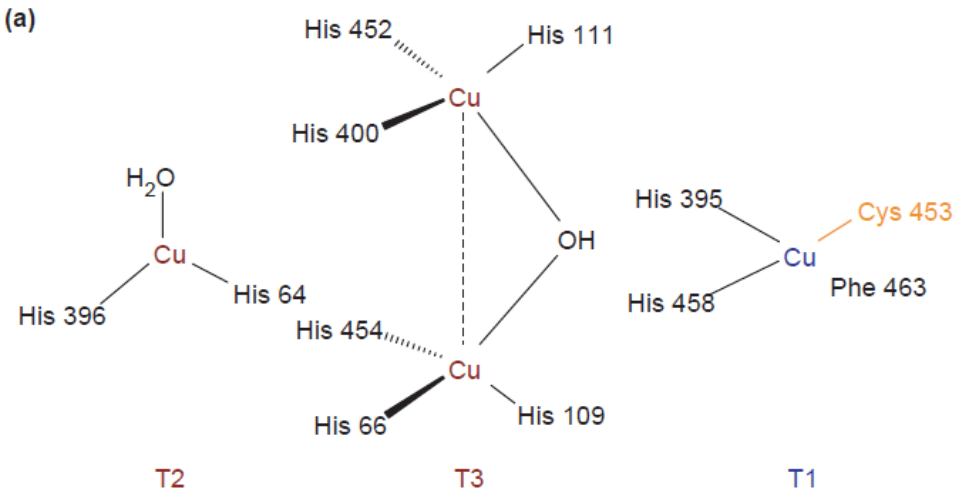


Figure 7: Structure of the catalytic cluster of the laccase from the white-rot fungus *trametes versicolor* [27].

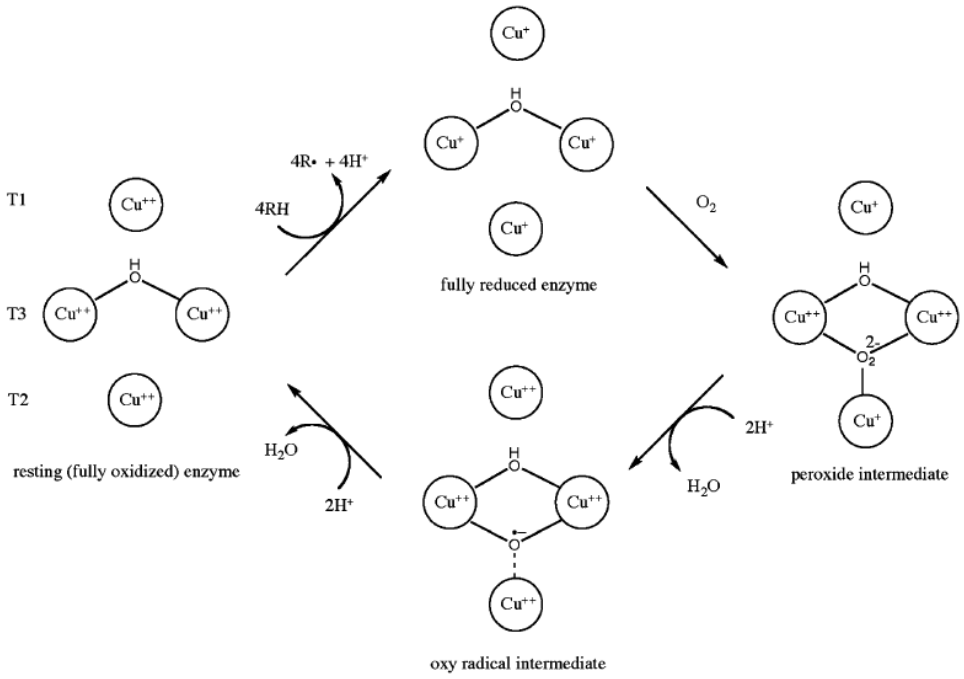


Figure 8: Mechanism of the catalytic cycle of laccase producing two molecules of water under one molecule reduction of atmospheric oxygen and simultaneous oxidation of four molecules substrate [28].

The catalytic efficiency of laccases depends on the redox potential of the T1 copper site, in the way that the higher the redox potential the higher the catalytic efficiency [29]. The redox potential of the T1 copper site varies depending on the origin of the laccase between about 400 to 800 mV [30]. In general, fungal laccases have higher redox potentials than bacterial or plant laccases, which is the reason for their preferred application in biotechnology [26][28].

In nature, laccases are widely distributed and play a role in multiple reactions [30]. In plants, the enzyme participates together with peroxidases in the biosynthesis of lignin [30]. Bacterial laccases are described to be involved in pigmentation and resistance of spores and pathogenesis [30]. Laccases of ligninolytic white-rot basidiomycetes have the ability to participate together with peroxidases and other enzymes in the degradation of lignin and detoxification of phenol compounds produced through degradation [31][30]. Laccases from fungal origin are the most common and most frequently described laccases in the literature [32]. These laccases from white-rot fungi are for example *Trametes versicolor*, *Trametes hirsuta, Trametes ochracea*, *Trametes villosa*, *Trametes gallica*, etc. [31][32].

The wide substrate range of laccases and low specificity towards their reducing substrates make them widely applicable for biotechnological purposes [30]. In addition, the use of molecular oxygen as electron acceptor and water as only by product of the reaction make them economic and environmental friendly [30]. Further, the application range of laccases has been expanded by the introduction of a laccase-mediator system (LMS), allowing the oxidation of non-phenolic compounds with a higher redox potential than laccases [30][31][32]. At the moment the main industrial applications of laccases are in the pulp and paper industry for the delignification of woody fibers during the bleaching process and in textile or dye industry, where laccases participate in the degradation of aromatic compounds. Another possible usage of the enzyme is the treatment of industrial wastewater. Here, laccases catalyze the oxidation of phenol derivates in wastewater, resulting in the formation of phenolic polymers, which then can be easily separated from the wastewater.[26][33]

The laccases used in this study are fungal laccases from *Trametes hirsuta* and *Myceliopthora thermophila*. *Trametes hirsuta* laccase (*Th*L) was purified from a white-rot basidiomycete fungus which causes wood decay [34]. The thermophilic fungus *Myceliopthora thermophila* secretes laccase and is common in high-temperature areas such as compost. In previous studies, the molecular mass of the used *Th*L was determined to be 62 kDa, it had an isoelectric point of pH 7, a pH optimum of 3.0 and an optimum temperature of 55°C [34]. *Trametes hirsuta* laccase was produced at the institute [34]. The laccase from thermophilic ascomycete *Myceliophthora thermophila* (*Mt*L) was purchased from Novozymes, Denmark. It is produced by fermentation of genetically modified *Aspergillus oryzae* and has been used for example for lignin degradation studies [35][36]. At the beginning of this study *Th*L and *Mt*L were characterized regarding their pH optimum and activity.

1.5 Laccase mediator system

Mediators are low molecular weight molecules, that act as electron shuttles and allow the oxidation of complex substrates, that otherwise couldn't be oxidized due to their high size or high redox potential. In other words by using a mediator the substrate range of laccases can be increased. As an example, during the degradation of lignin fungal laccases are able to directly oxidize phenolic units of lignin due to their low-redox potential. In contrast non-phenolic moieties of lignin, with redox potentials higher than the T1 sites of laccases (> 900mV), cannot be oxidized directly. However, due to the presence of mediators fungal laccases are able to indirectly oxidize non-phenolic compounds of lignin. Once the mediator is oxidized by the enzyme, the oxidized mediator diffuses away from the active site of the laccase and reacts in its oxidized state with the target substrate. Thereby, the mediator is reduced to its initial form and can participate again in the catalytic cycle. The proposed catalytic cycle of a laccase mediator system is illustrated in figure 9. [28][30][32][37]

In an ideal case, mediators are stable in their oxidized and reduced forms, are not inhibiting the enzyme and are fully recycled during the process. Also, ideal mediators are good laccase substrates. In practice, the majority of laccase mediators do not fulfill these ideals because their intermediate state is not stable and falls out of the reaction cycle [30][38]. These non-ideal mediators are better referred to as "laccase enhancers". In the literature, both terms are often confused, and the term "mediator" is in general used to describe both ideal mediators and laccase enhancers [38].

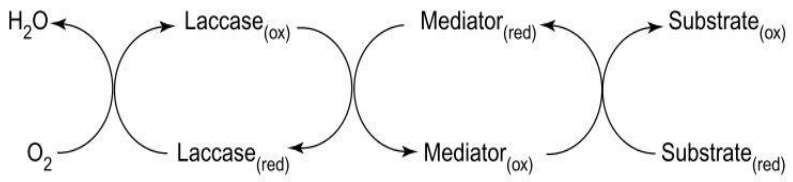


Figure 9: Proposed catalytic cycle of the laccase mediator system [27].

The synthetic mediators 2, 2'-azinobis-(3-ethylbenzothiazoline)-6-sulphonic acid (ABTS), 1-hydroxybenotriazole (HBT) can be described as being nearly ideal mediators, which is the reason for their wide application in biotechnological processes [27][31]. After ABTS has been first described as a laccase mediator for the oxidation of non-phenolic lignin model compounds by Bourbonnais and Paice in 1990 [39], a number of synthetic mediators have also been identified as laccase mediators (figure 10) [27]. Nevertheless, broad industrial applications of these synthetic mediators are restricted by high costs and possible toxicity [38]. That is why a lot of research groups focus on the search of alternative, natural laccase mediators [30][38][40]. Most of the identified natural laccase mediators are phenolic compounds related to the lignin polymer [30]. In figure 11 natural laccase

mediators are listed that have been tested to oxidize recalcitrant aromatic compounds. Part of this thesis was it, to identify laccase mediators next to ABTS and HBT, for the laccase mediator system catalyzed drying of alkyd resins.

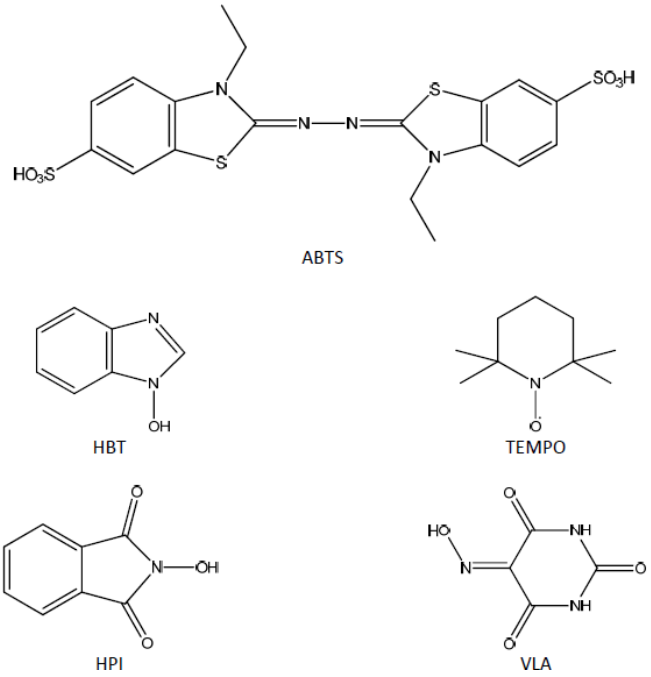


Figure 10: Structure of the synthetic mediators, 2, 2'-azinobis-(3-ethylbenzothiazoline)-6-sulphonic acid (ABTS), 1-hydroxybenzotriazole (HBT), 2,2,6,6-tetramethylpiperidin-1-yloxy (TEMPO), N-hydroxyphthalimide (HPI), violuric acid (VLA) [30].

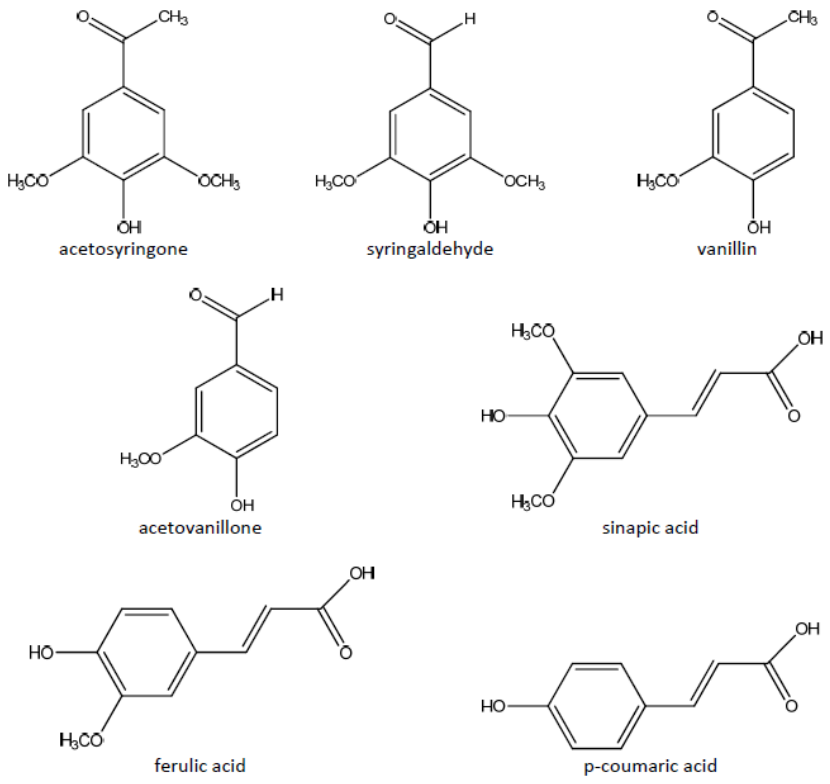


Figure 11: Chemical structure of naturally occurring phenolic compounds described as laccase mediators (acetosyrinone, syringaldehyde, vanillin, acetovanillone, sinapic acid, ferulic acid, p-coumaric acid) [30].

There are three different mechanisms of laccase mediator/enhancer systems described in the literature depending on the structure of the mediator [28][30][38][41]:

The laccase-ABTS catalyzed oxidation of non-phenolic lignin model compounds and organic dyes follows an **electron transfer (ET)** mechanism illustrated in figure 12. In the first stage ABTS is oxidized by the laccase to the intermediate cationic radical $ABTS^{+*}$. Further electron abstraction generates an $ABTS^{++}$ dication, which oxidizes the non-phenolic substrate. Mediators with the structure feature >N–OH, such as HBT, N-hydroxyphthalimide (HPI), violuric acid (VLA) or N-hydroxyacetanilide (NHA) react via an **hydrogen atom transfer (HAT)** mechanism (figure 12). The laccase oxidizes the mediator followed by immediate deprotonation generating nitroxyl radicals (N–O·). The target compounds are then oxidized by these extremely reactive radicals via H-atom abstraction. The laccase enhancers TEMPO and its analogues, where >N-O^{*} species are present, follow an **ionic oxidation mechanism**.

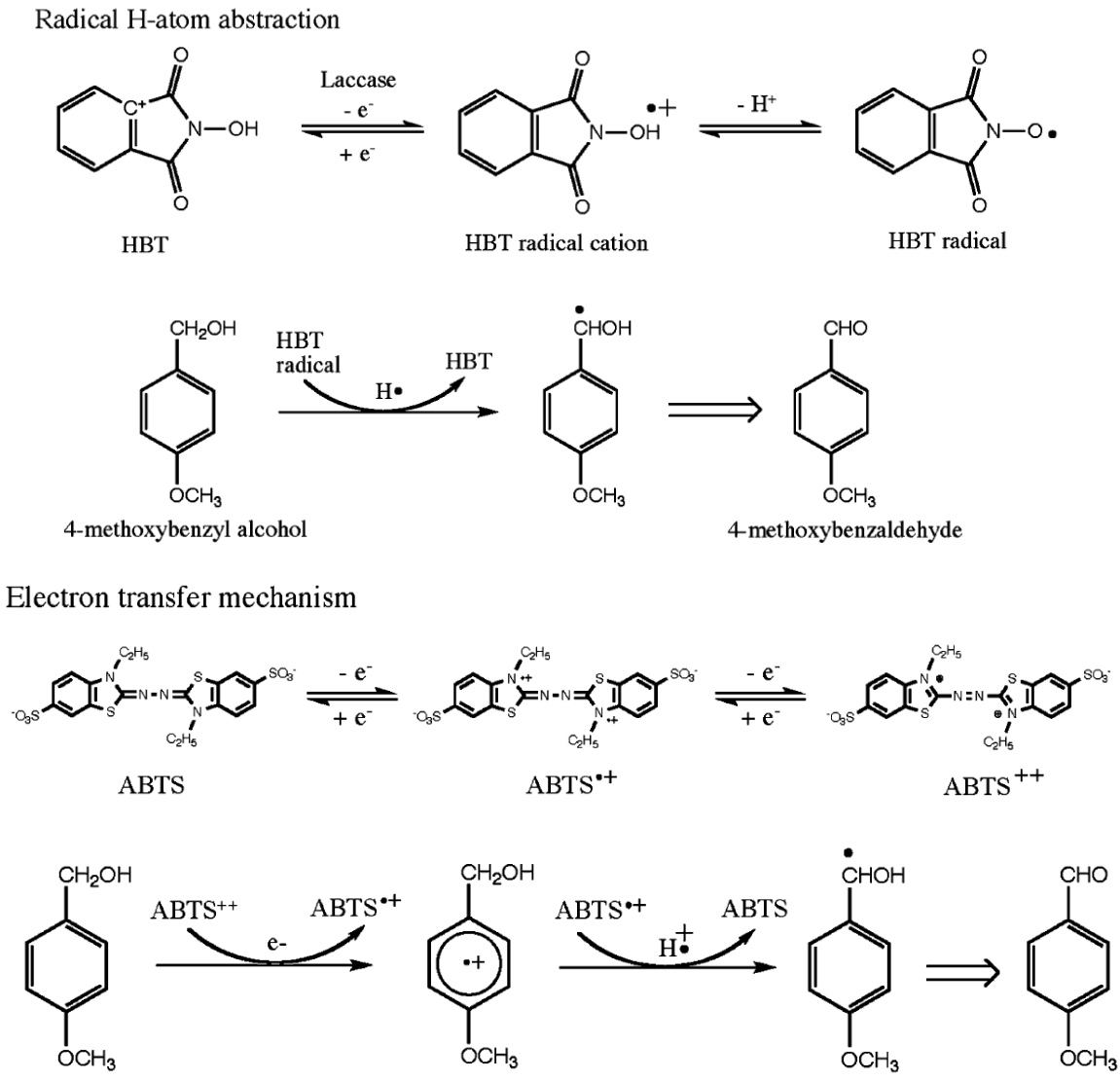


Figure 12: Illustration of the laccase mediated oxidation of a lignin model compound (p-ansilic alcohol), comparing two described oxidation mechanism, electron transfer mechanism (ET) and hydrogen atom transfer HAT [28].

1.6 Drying of alkyd resins catalyzed by a laccase mediator system

The capability of a laccase mediator system, to oxidize lipids and unsaturated fatty acids has been already established by some research groups [42][43][44][45]. In figure 13 a laccase mediator system catalyzed peroxidation of unsaturated fatty acids in lipids is shown for p-coumaric acid as natural phenolic laccase mediator (PhOH) [30].

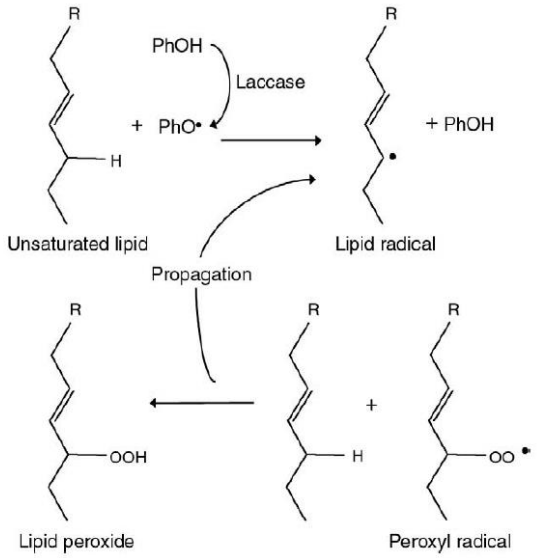


Figure 13: Peroxidation of unsaturated fatty acids in lipids produced by a laccase mediator system. The phenolic mediator p-coumaric acid (PhOH) is oxidized by the laccase forming phenoxyl radicals (PhO·) following a HAT mechanism [30].

In previous project studies it could be shown that a laccase mediator system (*Trametes hirsuta* laccase in combination with ABTS or HBT) catalyzes the cross-linking of unsaturated fatty acid side chains in alkyd resins [1]. Based on these studies, the following drying process of alkyd resin catalyzed by the LMS was suggested (figure 14) [1]. During the chemical cross-linking reaction the enzyme reduces oxygen to water, while the mediator is oxidized. The mediator reacts with the unsaturated fatty acid side chains of the alkyd resin and is then reoxidized by the laccase. It is supposed that the created fatty acid radicals then react with molecular oxygen and hydroperoxides are produced. The radical chain reaction proceeds by decomposition of the hydroperoxides and peroxy-radicals as well as alkoxy-radicals are formed. Recombination of the radicals results in carbon-carbon, peroxy and ether cross-linkages, leading to a three dimensional cross-linked polymer. [1][30]

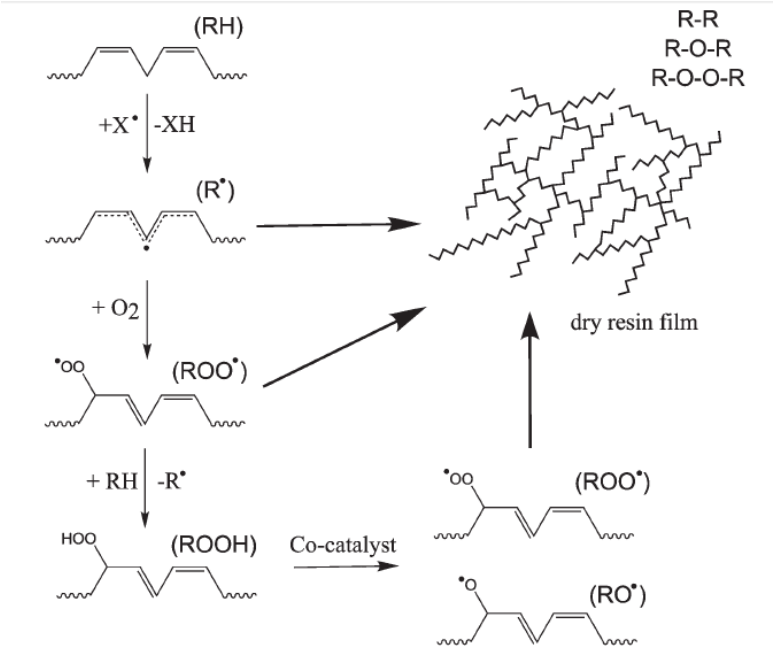


Figure 14: Hypothesized drying reaction of alkyd resins catalyzed by a laccase mediator system via peroxy-cross-linking [1].

2 Materials and Methods

2.1 Characterization of laccases

Two laccases from *Trametes hirsuta* (*Th*L) and *Myceliophthora thermophila* (*Mt*L) were analyzed regarding their activity and pH-optimum. Furthermore the protein concentration of the two laccase solutions was measured. The laccase from *Trametes hirsuta* was produced and purified as described by Almansa et al. [23]. The *Myceliophthora thermophila* laccase was supplied by Novozymes, Denmark, testing three batches of *Mt*L, with the stated numbers 51003, 42035, 40053.

2.1.1 Protein concentration

The concentration of the dissolved protein was determined using a Novagen® BCA Protein Assay Kit. The assay is based on a biuret reaction, whereby Cu^{2+} is reduced to Cu^{+} by proteins in an alkaline environment. Bicinchoninic acid forms with the reduced copper ions a purple chelate complex, which has a strong absorbance at 562 nm. The working range of this copper-based protein assay is 20 - 2000 µg/mL.

To determine the protein concentration of the laccase solutions, a calibration with bovine serum albumin (BSA) in a concentration range between 25 – 1000 µg/mL was done. The laccase solutions were diluted with ddH_2O to be in the range of the calibration curve. 25 µL of the BSA standard solution and the diluted laccase samples respectively were pipetted into the wells of a 96 well micro titer plate. 200 µL of BCA working agent were added and then incubated on a shaker for 30 minutes at 37°C. The absorption measurements were performed at the plate reader “TECAN infinitive M200” at 562 nm.

2.1.2 Laccase activity

The laccase activity was determined following the method developed by Liu et al [46] with some modifications. The assay is based on the generation of oxidized ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) by laccase, which forms a stable green radical dication and can be determined spectrophotometrically at 420 nm on the photometer. One unit (U) is defined as the amount of enzyme that catalyzes the oxidation of one micro mole of ABTS per minute.

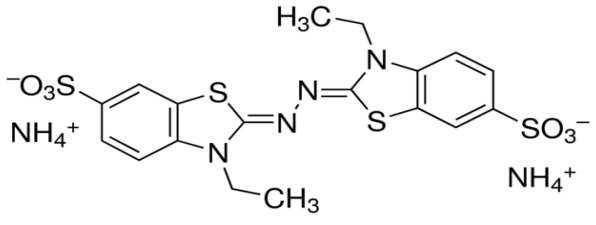


Figure 15: ABTS diammonium salt [47].

Before the activity measurement the molar extinction coefficient for ABTS at 420 nm at different pH values was determined using the Lambert-Beer law (equation 1).

Equation 1: Lambert-Beer law

$$E_{420} = \varepsilon_{ABTS} \cdot c_{ABTS} \cdot d \Longrightarrow \varepsilon_{ABTS} = \frac{E}{c_{ABTS} \cdot d} * f$$

E_{420}: absorbance at 420 nm
ε_{ABTS}: molar decadic absorption coefficient of ABTS at 420 nm and a certain pH value [L/(mmol*cm)]
c: molar concentration of the oxidized ABTS-solution [mol L^{-1}]
d: optical path length [cm], (1 cm)
f: dilution factor of the oxidized ABTS-solution

For the determination of the extinction coefficient, 5 mL ABTS solution (20 mM in ddH_2O) were oxidized with 100 µL *Mt*L (batch number 51003) for 64 hours at room temperature. The oxidized ABTS solution was diluted with 100 mM sodium acetate buffer pH 4, 4.5, 5 and 100 mM sodium phosphate buffer pH 6, 7, 8 in a concentration range of 0.2 – 0.005 mM. The absorbance of the diluted oxidized ABTS solutions was measured on the photometer at 420 nm. The measured absorbance was plotted against the known concentration of the ABTS dilutions. The gained slope represents the molar decadic absorption coefficient of ABTS at 420 nm and the used pH value.

To determine the activity of the laccase solutions, 650 µL of with buffer diluted enzyme were put into a cuvette. The reaction was started by adding 200 µL ABTS solution (10 mM in ddH_2O) and the absorbance change, caused by the oxidation of ABTS, was followed on the photometer at 420 nm (HITACHI U-2001) for one minute at standard conditions. The volumetric enzyme activity was calculated using equation 2.

Equation 2: Calculation of the volumetric enzyme activity.

$$v0 = \frac{\Delta abs}{\Delta t} = \frac{\Delta abs}{min} * \frac{Vtot}{Vsample * \varepsilon * d} * f$$

V_0: volumetric enzyme activity [U/mL]
Δabs/Δt: change of absorbance per minute [abs/min]
ε_{ABTS}: molar extinction coefficient of ABTS at 420 nm and a given pH value [L/(mmol*cm)]
V_{tot}: total volume [µL], (850 µL)
V_{sample}: sample volume [µL], (650 µL)
d: optical path length [cm], (1 cm)
f: dilution factor

2.1.3 pH Optimum

The optimal pH for the activity of each laccase was determined using the assay previously described in chapter 2.1.2. Therefore 100 mM sodium acetate buffer pH 4, pH 4.5, pH 5 and sodium phosphate buffer pH 6, pH 7 and pH 8 were used to dilute the laccase.

2.1.4 SDS-PAGE analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in order to estimate the molecular size of the *Mt*L. In previous studies the molecular size of *Th*L has been determined to be about 66 kDa by SDS-PAGE. The tested enzymes were the different batches of *Mt*L (42035, 51003 and 40035). The "PageRuler* Prestained Protein Ladder 10-170 kDa" from Thermo Scientific was used as a standard. The enzyme samples were diluted in ddH_2O. *Mt*L 42035 was diluted 1:2, *Mt*L 51003 1:10 and 1:50 and *Mt*L 40035 1:10 and 1:50. 20 µL of the dilutions, the pure enzyme sample of *Mt*L 42035 and the standard were mixed with 20 µL sample buffer respectively. The samples were heated at 95°C for 5 minutes and 10 µL of the denatured sample was loaded onto the gel. Separation was performed via gel electrophoresis at a charge of 100 V for 90 minutes. Proteins on the gel were stained with Coomassie Brilliant Blue for 60 minutes at room temperature. Finally the gel was incubated in destaining solution until proper bands were visibly.

Table 1: Composition of gels, buffers and solutions used for SDS-Page analysis.

Separating gel	40 % Acryl amide bisacrylamide	2.5 mL
	1.5 M Tris-HCl pH 8.8	2.5 mL
	ddH_2O	4.8 mL
	10 % SDS	0.1 mL
	10 % APS	0.05 mL
	TEMED	0.012 mL
Stacking gel	40 % Acryl amide bisacrylamide	0.5 mL
	0.5 M Tris-HCl pH 8.8	1.25 mL
	ddH_2O	1 mL
	10 % APS	0.025 mL
	TEMED	0.008 mL
Sample buffer	0.5 M Tris-HCl pH 8.8	1 mL
	ddH_2O	2.8 mL
	10 % SDS	3.2 mL
	Glycerol	1 mL
	1 % Bromphenol blue	0.5 mL
Running buffer (5x concentrated)	Tris	15 g
	Glycerol	72 g
	SDS	5 g
	ddH_2O	1000 mL
Staining solution	Coomassie Brilliant Blue	0.625 g
	Acetic acid	13.75 mL
	Ethanol	125 mL
	ddH_2O	250 mL
Destaining solution	ddH_2O	700 mL
	Acetic acid	150 mL
	Methanol	150 mL

2.2 Inhibition test of *Mt*L

One very important factor to reach a good drying performance is to adjust the pH of different alkyd resins to the evaluated pH optimum of the enzyme. For pH adjustment of the alkyd resin, N,N-Dimethylethylamine (DMEA) is added to the alkyd resin (long alkyd resin, UE-HSW 323/23c, inhibitor free). In order to reach the enzyme pH optimum of 7, a concentration of 0.08 % w/w DMEA in the long alkyd resin is required. To verify if DMEA has an inhibiting effect on the activity of *Mt*L, the following test was performed. The structure of DMEA can be found in figure 16.

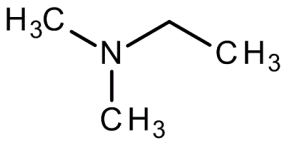


Figure 16: Structure of N,N-Dimethylethylamine [48].

The enzyme *Mt*L (batch 51003) was diluted 1:20000 in a 0.08 % DMEA solution in 100 mM sodium phosphate buffer (pH 7.4). Diluted enzyme was taken as a control and the samples were incubated at 25°C. At regular intervals enzyme activity was determined using the ABTS based activity assay, described in chapter 2.1.2.

2.3 Oxygen consumption measurement

During the cross-linking reaction oxygen as electron acceptor is reduced to water by laccases, while a substrate (e.g. fatty acid or mediator) is oxidized. This fact is used to follow the chemical crosslinking by measurements of the amount of consumed oxygen [1].

All the measurements are carried out in solution and a typical oxygen profile evaluation is shown in figure 17. Initially the mediator is dissolved in a buffer solution and put into a stirred beaker. In a first step laccase (black arrow) is added and oxidizes the mediator. Oxygen is consumed until the mediator is fully oxidized. Since this is an open experimental set-up, the oxygen concentration recovers and reaches a stable value again. The second step of the oxidation reaction is started by adding the substrate, in our case long alkyd resin (red arrow). The oxidized mediator reacts with the substrate, and is then reoxidized by the laccase. This second decrease of oxygen concentration is the starting point of the cross-linking reaction and indicates the efficiency of the LMS.

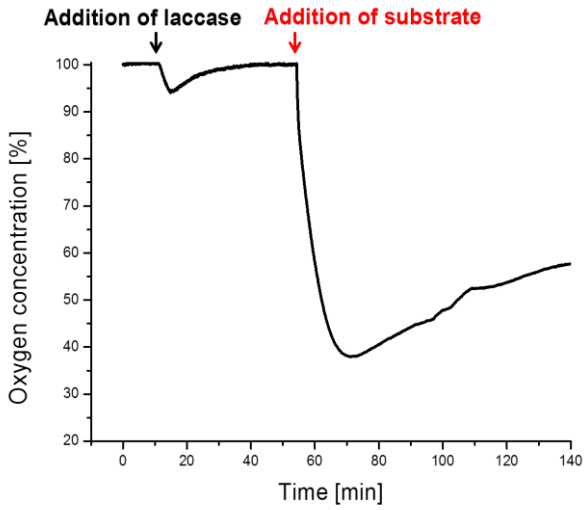


Figure 17: Principle of the oxygen consumption measurement. After the addition of laccase and substrate in the presence of mediator the oxygen concentration decreases.

In the present study oxygen consumption measurements are an important tool to evaluate the overall efficiency of the process. It is used to find potent laccase mediators, to compare different substrates or to determine the optimum pH for the LMS catalyzed cross-linking reaction of alkyd resins. The amount of oxygen consumed is measured using fiber-optical oxygen sensors, so-called optrodes. The principle is shortly described below.

Fiber – optic oxygen microsensor (optrode)

Optrodes are optical sensors to measure chemical species and are the optical analog to electrodes. In general optrodes are used to determine pH, gases (e.g. CO_2 and O_2) and various ionic species. The principle of optrodes is the interaction of an analyte with an indicator that changes the optical properties of the analyte. Those properties could be for example pH-dependent color changes of an acid-base indicator. In the case of oxygen optrodes, oxygen-quenchable fluorophores are used as indicators. The fluorescence of these indicators change due to interaction with O_2 that then can be measured [49].

For the measurements in this study a contactless fiber-optic oxygen sensor from PyroScience was used. It consists of a coated optical fiber connected to a PC-controlled oxygen meter, and an oxygen-sensitive indicator dye immobilized in a flexible polystyrene-foil.

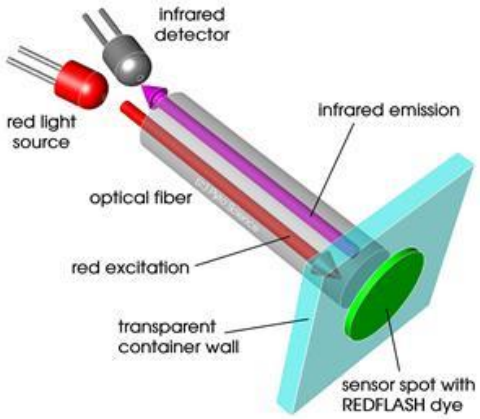


Figure 18: Sensor spots with an indicator dye are attached to the inner surface of the reaction vessel. The transmitter with its optical fiber is fixed opposite the sensor spot. The optical fiber is then connected to the PC-controlled oxygen meter [50].

The indicator dye is excited by red light with a wavelength of 610-630 nm and shows luminescence in the near infrared (NIR) at 760 – 790 nm. In the presence of oxygen, luminescence is quenched caused by an energy transfer, and the excess energy is transferred to the oxygen molecules. The quenching-degree corresponds to the oxygen concentration in the sample [50].

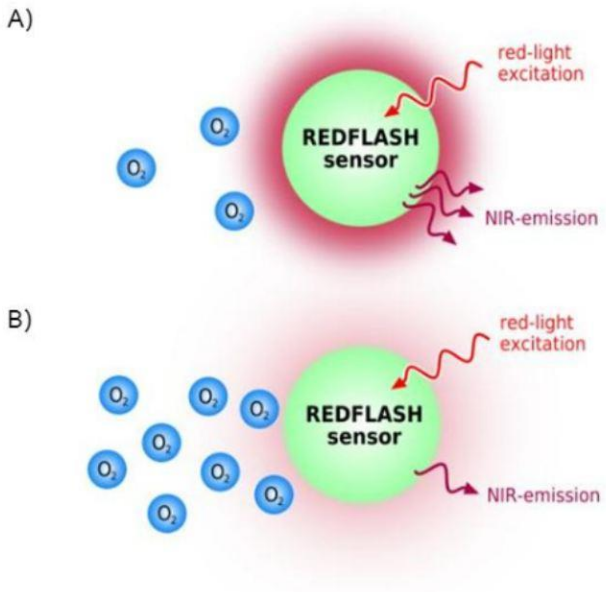


Figure 19: Principle of the measurement is based on oxygen caused fluorescence quenching. A) Red-light excitation of the REDFLASH sensor and emission in the near infrared. B) Decreased NIR-emission caused by the presence of oxygen [50].

Experimental set-up

1800 µL of a mediator dilution in buffer were put into a stirred glass beaker. As buffers 100 mM sodium acetate pH 4, 4.5 and 5 and 100 mM sodium phosphate pH 6, 7 and 8 were used. The enzymes, *Th*L and *Mt*L were diluted to have an activity of 5 U/mL or 10 U/mL in the corresponding buffer. 10 µL of enzyme dilution in buffer were added to the reaction. The enzyme is then present with an activity of 0.03 U/mL or 0.05 U/mL in the total reaction volume. After a certain time, the O_2

concentration was recovered and stable again. The second step of the reaction was started by adding 100 µL of long alkyd resin. In this study the alkyd resin (HSW323/23c, inhibitor free) will be called long alkyd resin. The consumption of oxygen was measured in duplicates, and the average value was calculated using the program OriginLab.

2.3.1 Inactive enzyme

As a negative control, *Th*L and *Mt*L have been inactivated prior to the oxygen consumption measurements. *Th*L lost its activity by adding NaF in a concentration of 79 mM and additional heating at 100°C for 30 minutes. *Mt*L was inactivated by heating at 100°C for 30 minutes. The loss of activity was determined using the ABTS activity assay described in chapter 2.1.2.

For the oxygen consumption measurements, a solution of 0.1 mM ABTS in buffer was prepared and put into the reaction vessel. Working with *Th*L a buffer with a pH value of 4.5, and for *Mt*L a buffer with pH 7 were used. 10 µL of inactivated enzyme and 100 µL of long alkyd resin were added.

2.3.2 pH profile

The oxygen consumption during the cross-linking reaction of long alkyd resin catalyzed by LMS was followed at different pH conditions to determine the optimal pH for the reaction. For the measurement, the enzymes *Th*L and *Mt*L were diluted to have an activity of 5 U/mL in the corresponding buffer and 10 µL of the diluted enzyme were added to the reaction. The mediator ABTS was diluted to a concentration of 0.1 mM in the respective buffer, and 100 µL of long alkyd resin was added as substrate.

2.3.3 Substrate screening

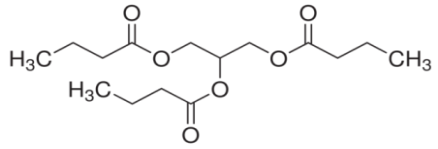
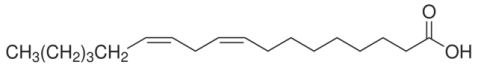
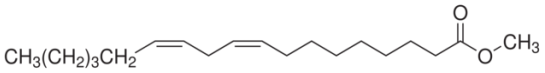
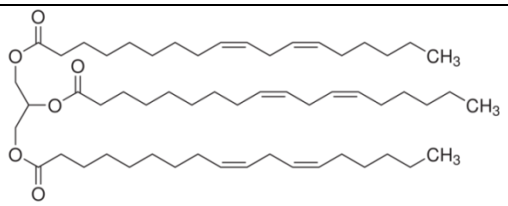
Different alkyd resins are having diverse types of oils in their formula depending on their final application. In this study a substrate screening was performed in order to determine the specificity of the enzyme towards the most common used oils in alkyd resins. For *Th*L, linseed oil was tested as substrate in buffer (pH 4) and a mediator concentration of 0.1 mM ABTS. During this work the focus was set on *Mt*L as laccase because on the one hand the pH of most commercial alkyd resins lies in the pH optimum of *Mt*L, on the other hand *Mt*L is easier commercially available compared to *Th*L. That is why the substrate screening was mainly performed with the enzyme *Mt*L and more substrates were tested for this enzyme, being linseed oil the only substrate tested for both enzymes. All tested substrates and their compositions are summarized in table 2.

All measurements were performed using a concentration of 0.1 mM ABTS dilution in buffer (pH 7) and 0.03 U/mL *Mt*L in the total reaction volume. With the substrates number 1-5, emulsions in water

were prepared, which consisted of 62 % substrate, 3 % emulsifier and 35 % dH_2O. The percentage of fatty acids in linseed oil, castor oil based alkyd resin and long alkyd resin can be found in table 2.

The substrates methyl linoleate, trilinolein, castor oil based alkyd resin, long alkyd resin and linseed oil were further investigated by using higher concentrations of *Mt*L (0.05 U/mL in total volume) and mediator (1 mM ABTS in buffer).

Table 2: Tested substrates and their structures. For linseed oil and the tested alkyd resins their fatty acid content is given.

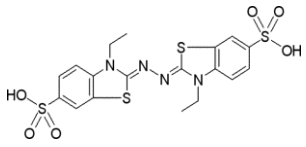
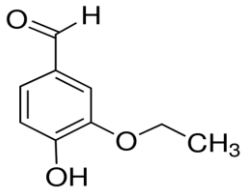
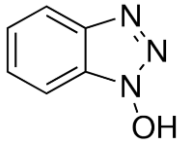
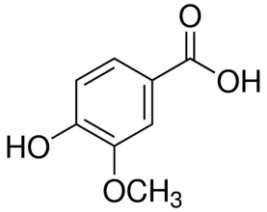
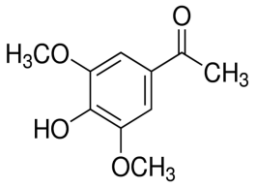
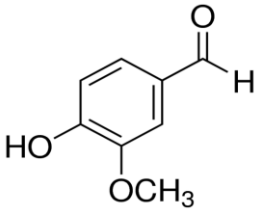
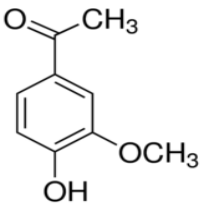
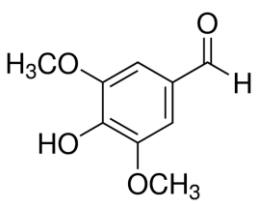
Nr.	Substrate	Structure	
1	**Tributyrin**	 [51]	
2	**Linoleic acid**	 [52]	
3	**Methyl linoleate**	 [53]	
4	**Trilinolein**	 [54]	
		Fatty acid precentages	
5	**Linseed oil**	Saturated fatty acids Oleic acid Linoleic acid (isolated) Linolenic acid	9 - 12 % 12 – 25 % 14 – 26 % 40 – 60 %
6	**Castor oil based alkyd resin (PRE6/15)**	Tall oil fatty acids Deyhdrated rhizinoleic fatty acid Linseed oil fatty acid	0.66 % 40.1 % 1.18 %
7	**Long alkyd resin (HSW 323/23c)**	Tall oil fatty acids Pretreated linseed oil Linseed oil fatty acid	0,64 % 40,52 % 1,15 %

2.3.4 Screening for laccase mediators

To screen for effective laccase mediators for the LMS catalyzed drying of alkyd resins, oxygen consumption measurements were performed. In previous project studies the synthetic mediators ABTS and HBT proved themselves as effective laccase mediators. In this study a laccase mediator screening was performed in order to identify effective, harmless and low-cost substances as laccase mediators using oxygen consumption measurements, drying time recorder measurements and FTIR spectroscopy.

For testing ethyl vanillin, vanillic acid, vanillin, syringaldehyde, acetosyringone and acetovanillone as potential laccase mediators, higher enzyme concentrations were used than for the previous performed oxygen consumption measurements. The enzyme *Mt*L was diluted to a final activity of 32 U/mL in a sodium phosphate buffer pH 7, and 10 µL of the diluted enzyme were added to the reaction. Hereby, *Mt*L was present with an activity of 0.2 U/mL in the total volume of the reaction. The studied mediators were diluted to 0.1 mM in buffer (pH7) and are listed in table 3. Oxygen consumption measurements were performed in duplicates using long alkyd resin as substrate.

Table 3: Tested synthetic and natural mediators for *Mt*L.

<table>
<tr><th colspan="4">Synthetic mediators</th></tr>
<tr><td>ABTS,
2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate)</td><td> 
[55]</td><td>Ethyl vanillin,
3-Ethoxy-4-hydroxybenzaldehyde</td><td rowspan="2"> 
[56]</td></tr>
<tr><td>HBT,
1-Hydroxybenzotriazole</td><td> 
[57]</td></tr>
<tr><th colspan="4">Natural mediators</th></tr>
<tr><td>Vanillic acid,
4-Hydroxy-3-methoxybenzoic acid</td><td> 
[58]</td><td>Acetosyringone
3′,5′-Dimethoxy-4′-hydroxyacetophenone</td><td> 
[59]</td></tr>
<tr><td>Vanillin,
4-Hydroxy-3-methoxybenzaldehyde, Vanillic aldehyde</td><td> 
[60]</td><td rowspan="2">Acetovanillone
4′-Hydroxy-3′-methoxyacetophenone</td><td rowspan="2"> 
[61]</td></tr>
<tr><td>Syringaldehyde,
4-Hydroxy-3,5-dimethoxybenzaldehyde</td><td> 
[62]</td></tr>
</table>

2.4 Testing the drying performance

The drying process was studied by drying time recorder analysis, a widely used and accepted method in the coatings industry. In previous studies it could already be shown that the LMS catalyzes the cross-linking reaction of alkyd resins. It was proven that the enzyme reaction works not only in aqueous media, but also in drying alkyd resin films [1]. In this study drying time recorder (DTR) measurements are used to determine the drying performance of different mediators and to investigate the storage stability of mediator and laccase in the long alkyd resin.

2.4.1 Principle of drying time recorder measurement

For the drying time recorder measurements coatings are applied on a glass stripe with a defined film thickness. A needle is then moved along the glass stripe with a defined velocity, scratching the surface of the film. Depending on the drying stage of the film, typical stages can be observed over time. The defined stages in the drying process, visualized in figure 20 are 1) Leveling 2) Basic trace 3) Ripped film 4) Surface trace and 5) Dry.

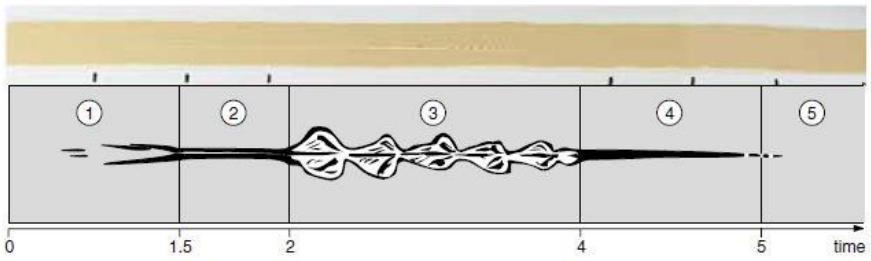


Figure 20: Typical stages of drying in coated films [63].

2.4.2 Screening for laccase mediators

The listed mediators in table 4 were investigated regarding their drying ability using DTR measurements. These substances were tested previously in oxygen consumption measurements, where all of them, except vanillic acid, turned out to be potential laccase mediators, judging by the consumption of oxygen over time. Additionally ABTS was tested in different concentrations and cobalt was used as a positive control.

First, the pH of the long alkyd resin was adjusted to 7 by adding DMEA. As a positive control, cobalt was mixed into the long alkyd resin in a concentration of 200 ppm. Mediator and laccase were added to the long alkyd resin adjusting to the final concentration listed in table 4. The mediators ABTS, vanillin and acetovanillone are soluble in water and therefore 20 mM ABTS, 60 mM vanillin and 30 mM acetovanillone solutions in dH_2O were prepared. With the other mediators a 5 % (w/w) mixture in emulsifier was made, because of their low solubility in dH_2O. The alkyd resin mixtures were stored 24 hours at room temperature, to give the catalysts time to diffuse into the alkyd resin.

As soon as the samples were applied on a glass stripe with a film thickness of 152 µm, drying time recorder measurements were started. Additionally the drying performance of samples marked in the table by an asterisk was analyzed via FTIR spectroscopy, described in chapter 2.6.1.

Table 4: Sample preparation for drying time recorder measurements and FTIR analysis. The samples marked with * have been additionally analyzed via FTIR spectroscopy.

Nr.	Catalyst	Concentration [ppm]		Long alkyd resin [g]			
1	Cobalt	200		6.055			
Nr.	**Mediator**	**Mediator concen-tration [mM]**	**Laccase concen-tration [U/mL]**	**Mediator solution [µL]**	**5 % mediator – emulsifier mixture [g]**	**Laccase [µL]**	**Long alkyd resin [g]**
2*	ABTS	1	8	315		27	6.030
3*	ABTS	2	8	665		28	6.025
4	ABTS	0.1	44	31		143	6.011
5*	ABTS	1	44	321		150	6.015
6*	ABTS	2	44	677		158	5.997
7*	Vanillin	2	8	207		26	6.030
8	Vanillin	4	8	425		27	5.995
9*	Vanillin	0.1	44	10		142	5.998
10*	Vanillin	2	44	210		147	6.019
11*	Vanillin	4	44	436		153	6.019
12*	Vanillin	8	44	939		164	6.006
13*	Acetovanillone	2	8	425		27	5.991
14*	Acetovanillone	2	44	435		152	6.006
15*	Acetovanillone	4	44	938		164	5.998
16*	Acetosyringone	2	44		0.05	144	6.030
17*	Ethyl vanillin	2	44		0.05	168	7.056
18*	Syringaldehyde	2	44		0.04	143	6.025
19*	Vanillic acid	2	44		0.04	143	5.997

2.5 Storage stability test

It could be observed that the drying performance, after the storage of laccase and mediator in the long alkyd resin, decreases. To investigate this fact drying time recorder and oxygen concentration measurements were performed. Therefore, laccase and mediator were stored separately in the long alkyd resin (adjusted to pH 7) and the missing compound was added 24 h before the measurement. The results were compared with long alkyd resin, in which laccase and mediator were stored together over time. The listed samples in table 5 were prepared and stored at room temperatures.

Table 5: Alkyd resin mixtures prepared to study the storage stability of laccase and mediator.

Sample	Stored substances	Long alkyd resin [g]	5 % (w/w) ABTS [g]	5 % (w/w) HBT [g]	*Mt*L 40035 [g]
1	*Mt*L	25			0.3
2	ABTS/HBT	25	0.29	0.29	
3	*Mt*L + ABTS/HBT	25	0.29	0.29	0.3

2.5.1 Drying time recorder measurement

After storage of 3, 5, and 25 days the stored long alkyd resin mixture of mediator and laccase (sample 3) was directly applied on a glass stripe and the drying performance was studied, following the method described in chapter 2.1.1. The long alkyd resin stored with *Mt*L (sample 1) was treated with 0.29 g ABTS and HBT respectively and 0.3 g *Mt*L (40035) was added to sample 2. Sample 1 and 2 were analyzed after a storage time of 25 days at room temperatures. The film thickness applied on the glass stripe was 152 µm.

2.5.2 Oxygen consumption measurement

1782 µL 50 mM ammonium acetate buffer (pH 7) were put into a stirred glass beaker and 100 µL stored long alkyd resin-laccase mixture (sample 1) were added. After the oxygen concentration reached a stable value, the reaction was started by adding 18 µL ABTS solution (10mM in ddH_2O). The measurements were performed after storage of 3, 10, 17 and 25 days at 25°C.

2.6 Process monitoring via FTIR spectroscopy

The drying process of alkyd resins or model compounds can be characterized on a molecular level using FTIR spectroscopy [64]. It was proven previously that both cobalt and LMS catalysts decrease the band assigned to double bonds in 3010 cm^{-1} and increase the region between 3100-3600 cm^{-1}, which is corresponding to OH containing compounds like hydroperoxides, alcohols, and acids [1].

2.6.1 Screening for laccase mediators

The mediators tested with oxygen consumption and drying time recorder measurements were additionally tested by FTIR analysis in order to get a deeper look on the drying potential. Therefore the listed samples (table 4) marked with an asterisk *, which have been also tested by DTR measurements, were chosen to be analyzed. The sample preparation is described in chapter 2.4.2. For the measurement, the long alkyd resin samples were applied on a CaF_2 crystal window with a film thickness of 30 µm. Measurements were done over time on the FTIR spectrometer Spectrum 100 from Perkin Elmer. For each spectrum three scans with a resolution of 4.00 cm^{-1} were taken.

2.7 Analysis of cross-linked linseed oil and alkyd resin

Unsaturated-fatty acid moieties in the long alkyd resin and linseed oil were cross-linked by LMS and further investigated by analyzing the hereby formed polymers. Therefore, the reaction mixtures, containing the substrate, mediator and laccase, were lyophilized after a certain incubation time and the formed solid residue was studied by ATR-FTIR spectroscopy and gas chromatography. The sample preparation and the used concentrations of mediator and laccase are based on the experimental set-up of the oxygen consumption measurements in solution described in chapter 2.3. Hence, 1800 µL mediator buffer solution were put into a stirred glass beaker, 10 µL diluted enzyme solution and 100 µL substrate were present in the reaction mixture. The reaction mixtures were stirred for 24 h or 48 h time, then frozen in liquid nitrogen and lyophilized overnight. The samples for GC and FTIR analysis were treated in the same manner, as shown in the scheme of the sample preparation in **figure 21**figure 21.

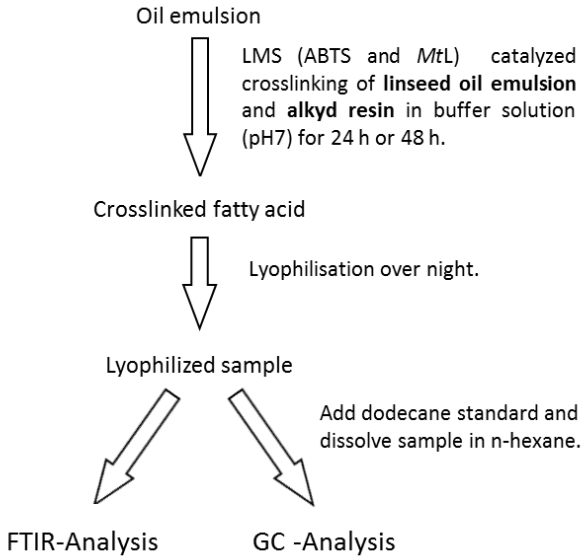


Figure 21: Schematic illustration of the sample preparation for analysis via ATR-FTIR and gas chromatography.

2.7.1 ATR-FTIR spectroscopy

For the cross-linking reaction 1800 µL 0.1 mM ABTS solution in 50 mM ammonium acetate buffer (pH7) and 10 µL of *Mt*L diluted to 5 U/mL were used. As substrate 100 µL of emulsified linseed oil or long alkyd resin was added. The samples were stirred for 24 h or 48 h, and the lyophilized solid residues were studied using ATR-FTIR spectroscopy, taking three scans for each spectrum with a resolution of 4.00 cm^{-1}.

2.7.2 Gas chromatography

During the chemical drying of alkyd resins, the unsaturated fatty acid moieties are oxidized and subsequently cross-linked [12], leading to a reduction of the free triglyceride content. Since alkyd

resins are a very complex system, linseed oil was used as a model substrate. Sample preparation for GC analysis, was done like shown in figure 21 - to the lyophilized sample dodecane was added as internal standard and n-hexane was used as solvent.

Linseed oil emulsion

The samples analyzed via gas chromatography are listed in table 6. For the reaction 1800 µL 0.1 mM or 1 mM mediator solutions in 50 mM ammonium acetate buffer (NH_4Ac, pH7) were used, adding 10 µL laccase with an activity of 5 U/mL or 10 U/mL and 100 µL emulsified linseed oil. The enzyme activity in the total reaction volume is given in table 6. The 0 hours samples were frozen immediately after mixing, while the other samples were stirred for 24 h before lyophilization. As controls, samples without enzyme and mediator were used, as well as samples where either mediator or laccase is missing. To the lyophilized samples 100 µL of a 10 % dodecane standard and 900 µL n-hexane were added. The analysis was performed using GC-FID.

Table 6: Preparation of the samples for GC-analysis using linseed oil as a model substance.

Sample name	Reaction time	Content		
A1-L10-0h-1	0 h	1 mM ABTS in NH_4Ac	0.05 U/mL MtL	100 µL linseed oil emulsified (62 % oil, 3 % emulsifier, 35 % water)
A1-L10-0h-2				
A1-L10-0h-3				
A1-L10-24h-1	24 h			
A1-L10-24h-2				
A1-L10-24h-3				
A01-L5-0h-1	0h	0.1 mM ABTS in NH_4Ac	0.03 U/mL MtL	
A01-L5-0h-2				
A01-L5-0h-3				
A01-L5-24h-1	24 h			
A01-L5-24h-2				
A01-L5-24h-3				
0-0-0h-1	0	NH_4Ac	no enzyme	
0-0-0h-2				
0-0-24h-1	24h			
0-0-24h-2				
A1-0-0h-1	0	1 mM ABTS in NH_4Ac	no enzyme	
A1-0-0h-2				
A1-0-24h-1	24h			
A1-0-24h-2				
0-L10-0h-1	0	NH_4Ac	0.05 U/mL MtL	
0-L10-0h-2				
0-L10-24h-1	24h			
0-L10-24h-2				

GC Settings: The inlet temperature was adjusted to a temperature of 350°C, with a split ratio of 10:1 and a helium flow rate of 3 mL/min. The GC-column temperature was kept at 50°C for one minute and raised to 380°C at a rate of 10°C/min and maintained at 380°C for 5 minutes.

Prior to the samples the standards dodecane, linoleic acid and trilinolein were analyzed. Therefore 2 µL of the standard was diluted in 998 µL n-Hexane. For the linoleic acid standard the inlet temperature was lowered to 280°C, the other settings did not change.

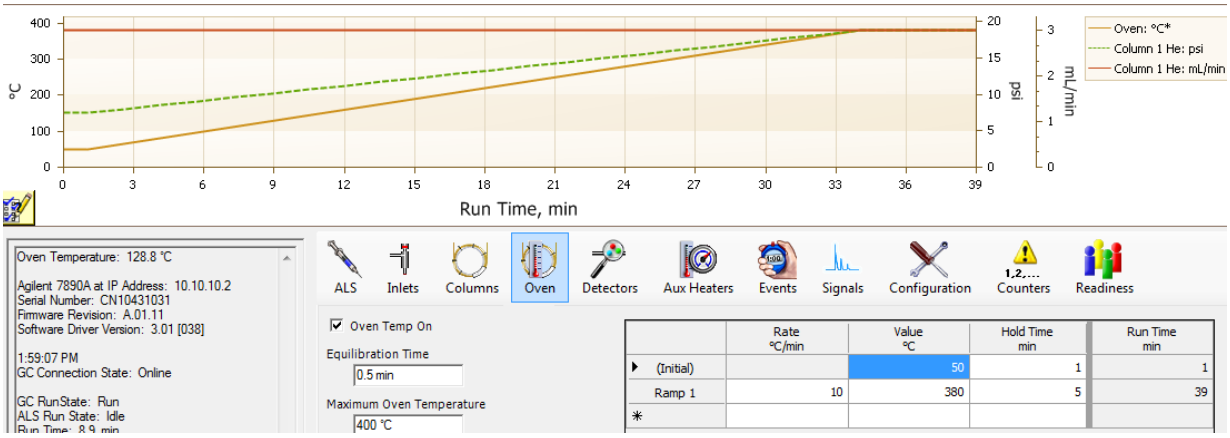


Figure 22: Temperature gradient in the GC oven. Additionally the carrier gas flow is shown, which remains constant while the pressure rises due to the temperature changes.

Alkyd resin

For the reaction with 100 µL long alkyd resin, 10 µL of the enzyme with an activity of 5 U/mL was added to 1800 µL of a 0.1 mM mediator solution in 50 mM ammonium acetate buffer (NH_4Ac, pH7). The settings of the GC-analysis and sample preparation were the same as the measurements of the linseed oil samples. A summary of the analyzed samples with the used concentrations of mediator and enzyme in the total reaction volume can be found in table 7.

Table 7: Content and reaction time of alkyd resin samples prepared for GC-analysis. The activity of *Mt*L relates to the activity in the total reaction volume.

Sample name	Reaction time	Content		
A01-L5-0h-1	0h	0.1 mM ABTS in NH_4Ac	0.03 U/mL *Mt*L	100 µL long alkyd resin
A01-L5-0h-2				
A01-L5-0h-3				
A01-L5-24h-1	24 h			
A01-L5-24h-2				
A01-L5-24h-3				
0-0-0h-1	0	NH_4Ac	no enzyme	
0-0-0h-2				
0-0-24h-1	24h			
0-0-24h-2				
A01-0-0h-1	0	0.1 mM ABTS in NH_4Ac	no enzyme	
A01-0-0h-2				
A01-0-24h-1	24h			
A01-0-24h-2				
0-L5-0h-1	0	NH_4Ac	0.03 U/mL *Mt*L	
0-L5-0h-2				
0-L5-24h-1	24h			
0-L5-24h-2				

3 Results

3.1 Characterization of the Laccases

The aim of this project is to substitute conventional cobalt-based driers in alkyd resin coatings by a laccase mediator system (LMS). In previous project studies it was proven that a LMS efficiently catalyzes the cross-linking reaction [1]. To be able to optimize the LMS reaction, a detailed analysis of the oxidative reaction processes is necessary. The first step to achieve this goal is to characterize the laccases regarding their pH optimum and activity. The two laccases that were used in this study originated from *Trametes hirsuta* (*Th*L) and *Myceliophthora thermophila* (*Mt*L) and both were characterized and tested for their drying ability in alkyd resin paints. Furthermore the protein concentration of the two used laccase solutions was measured. *Myceliophthora thermophila* laccase was available in three batches, which were characterized individually.

3.1.1 Protein concentration

The protein concentration of the laccases was measured using a BCA protein assay, using bovine serum albumin as a standard. The calibration curves for the determination of the protein concentrations of *Trametes hirsuta* laccase and *Myceliophthora thermophila* laccase can be found in figure 23 and figure 24.

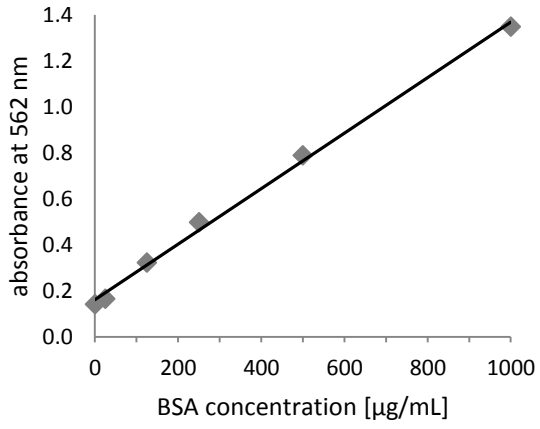


Figure 23: BSA calibration curve for the determination of the protein concentration of *Trametes hirsuta* laccase.

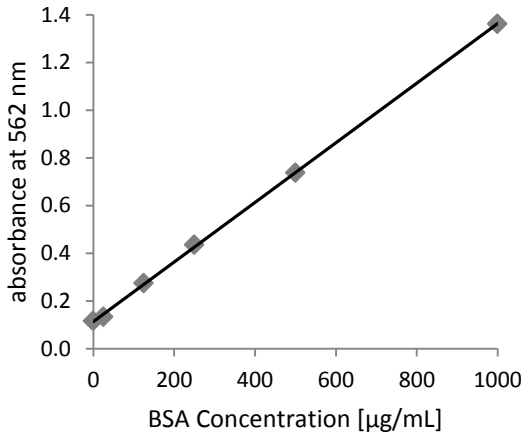


Figure 24: BSA calibration curve for the determination of the protein concentration of *Myceliophthora thermophila* laccase.

The protein concentration of the laccase was calculated with equation 3. The calibration functions are listed in table 8.

Equation 3: Calculation of the protein concentration of the laccase.

$$c_{Protein} = \frac{abs[562] - d}{k} \cdot f$$

$c_{Protein}$	protein concentration of the sample [µg/mL]
abs[562]	measured extinction on the plate reader at 562 nm
d	blank value
k	gradient of the calibration curve
$f_{dilution}$	dilution coefficient of the sample

Table 8: Calibration functions to determine the protein concentration of the laccase.

Calibration	k (µg mL^{-1})	d	R^2
Trametes hirsuta laccase	0,001	0,161	0,997
Myceliophthora thermophila laccase	0,001	0,114	0,999

The determined protein concentrations of the tested laccase solutions are listed in table 9. The *Mt*L batch 42035 was identified to have the lowest protein concentration.

Table 9: Protein concentrations of *Trametes hirsuta* laccase and *Myceliophthora thermophila* laccase (51003, 42035, 40035).

Enzyme	Batch number	Protein concentration [µg/mL]
Trametes hirsuta laccase		3070 ± 300
Myceliophthora thermophila laccase	51003	53960 ± 2300
	42035	2000 ± 150
	40035	51500 ± 1600

3.1.2 Laccase activity

The enzyme activity of the two laccases was determined measuring the oxidation rate of ABTS. In its oxidized state ABTS forms a stable green radical dication, which can be measured spectrophotometrically. The formation rate of the oxidized ABTS at 25 °C and a given pH was monitored on the photometer at 420 nm to determine the laccase activity. The amount of enzyme needed to oxidize 1 µM of ABTS per minute is defined to be one unit of laccase activity.

In general, the method to determine the laccase activity using an ABTS assay is a rapid, simple and well known approach [65]. However, the artificial, substrate free condition might not adequately mimic the conditions found in the drying alkyd resin film [65]. A good way to observe the overall process of cross-linking is to measure the oxygen consumption during the process. The results of the oxygen consumption measurements can be found in chapter 3.3.2.

The molar extinction coefficient of ABTS at 420 nm

Prior to laccase activity measurements, the molar extinction coefficient of ABTS at 420 nm and different pH conditions was determined. The molar extinction coefficient of ABTS is needed to calculate the activity of the laccase solutions using the Lambert Beer law (equation 2). Therefore, a fully oxidized ABTS solution was diluted in buffer solutions with various pH values. The absorbance of the diluted oxidized ABTS solution was then measured at 420 nm on the photometer.

The results of the measurements can be found in figure 25. By plotting the measured absorbance of the oxidized ABTS solution against the known concentration, a slope is gained for each pH value. Each slope represents the molar decadic absorption coefficient of ABTS at 420 nm and the stated pH-value. In view of the performed measurements, the molar decadic coefficient of ABTS depends strongly on the pH conditions. This strong pH-dependency has to be taken into account for the determination of the laccase activity using an ABTS assay.

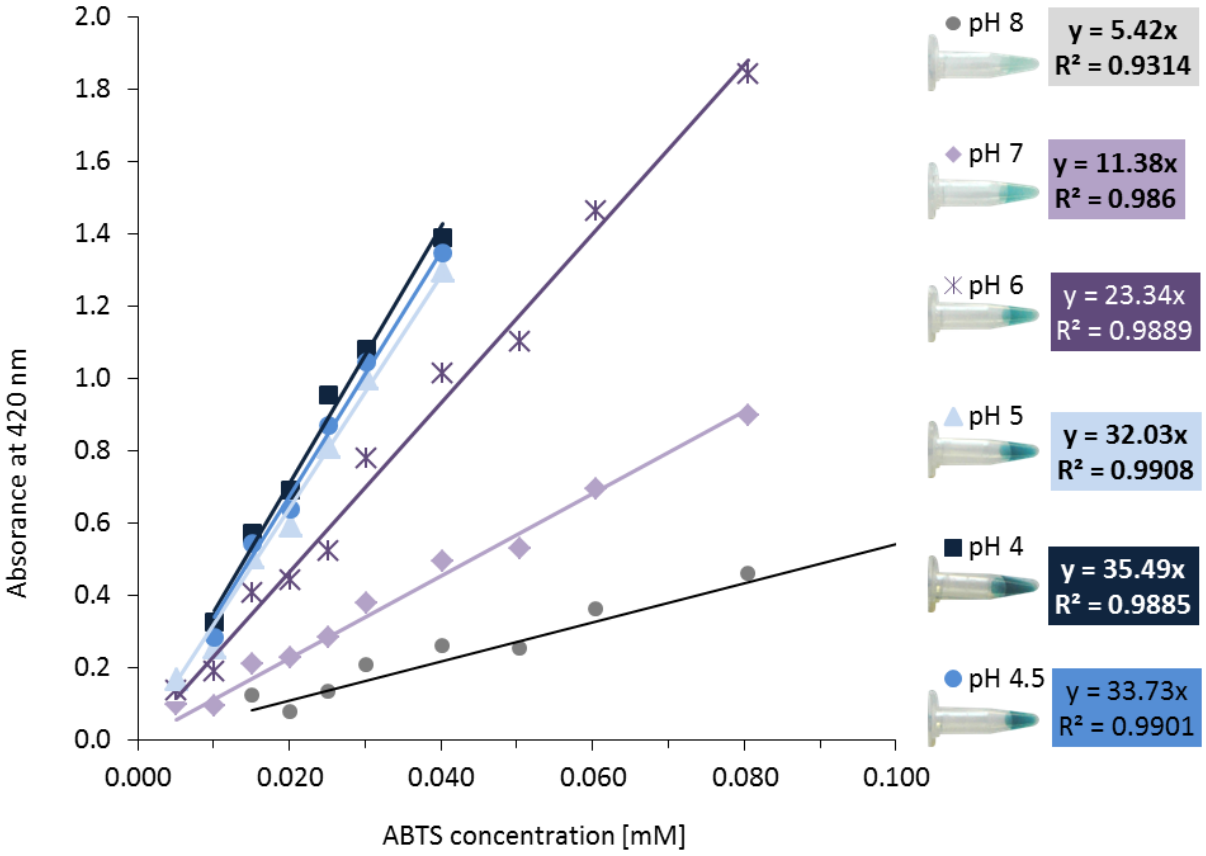


Figure 25: pH dependency of the molar decadic absorption coefficient of ABTS at 420 nm. The measured absorbance at different pH conditions at 420 nm is plotted against the ABTS concentration, in order to determine the molar decadic absorption coefficient of ABTS. The slope represents the molar decadic absorption coefficient of ABTS at 420 nm and the stated pH value. The linear equation for each slope can be found next to the given pH value. The color differences of the oxidized ABTS solutions at different pH conditions are clearly visible.

To give an overview the pH dependent molar decadic absorption coefficients of ABTS at 420 nm are listed in table 10.

Table 10: Molar decadic absoption coefficients of ABTS at 420 nm and stated pH conditions.

pH	ε [L/mmol * cm]
4	35.49
4.5	33.73
5	32.03
6	23.34
7	11.38
8	5.42

3.1.3 pH optimum

The effect of the pH on the activity of *Trametes hirsuta* laccase and *Myceliophthora thermophila* laccase was determined by laccase activity measurements and the pH optimum profiles for the two laccase types are shown in figure 26 and figure 27. For *Mt*L three different batches were analyzed and the calculated specific activities of *Mt*L and *Th*L can be found in figure 53 in the appendix.

Table 11: pH dependent activities [U/mL] of *Trametes hirsuta* laccase and *Myceliophthora thermophila* laccase.

Enzyme	Buffer	pH	Activity [U/mL]
Trametes hirsuta laccase	100 mM sodium acetate	4	25.7 ± 0.6
		4.5	21.7 ± 2.3
		5	16.9 ± 1.0
	100 mM sodium phosphate	6	8.2 ± 1.6
		7	0.9 ± 0.1
Myceliophthora thermophila laccase, Batch number - 51003	100mM sodium acetate	3	607 ± 21
		4	600 ± 4.0
		4.5	497 ± 8.0
		5	504 ± 9.0
	100mM sodium phosphate	6	1067 ± 4.0
		7	1303 ± 7.0
		8	651 ± 24
Myceliophthora thermophila laccase, Batch number - 42035	100mM sodium acetate	4	17.3 ± 0.5
		4.5	14.9 ± 0.6
		5	17.6 ± 1.3
	100mM sodium phosphate	6	87.1 ± 7.2
		7	60.4 ± 0.3
		8	36.4 ± 3.5
Myceliophthora thermophila laccase, Batch number - 40053	100mM sodium acetate	4	549.8 ± 19
		4.5	466.7 ± 20
		5	696.2 ± 5.7
	100mM sodium phosphate	6	733.7 ± 34.5
		7	1361.7 ± 11.5
		8	488.3 ± 20.2

The pH of the tested long alkyd resin is set to pH 7 by adding dimethylethanolamine. During the drying process, the added dimethylethanolamine evaporates, which causes a shift of the pH in the drying long alkyd resin film. Therefore, it is of high importance that the used laccase covers a broad pH spectrum. Both *Mt*L and *Th*L cover a broad pH spectrum with different pH optimums. The highest activity for *Th*L was measured at pH 4. *Mt*L was most active at pH 7 or pH 6, depending on the batch of the enzyme. The pH optimum of *Mt*L (batch 40035 and 51003) is pH 7, which makes it ideal for an application in alkyd resin paints. Batch number 42035 has significant lower activities than the batches 40035 and 51003. This can be explained by the lower protein concentration of this batch and is the reason why it was not used for further drying tests. All in all, laccase activity measurements showed that *Mt*L has a higher specific activity than *Th*L at their respective pH optimum.

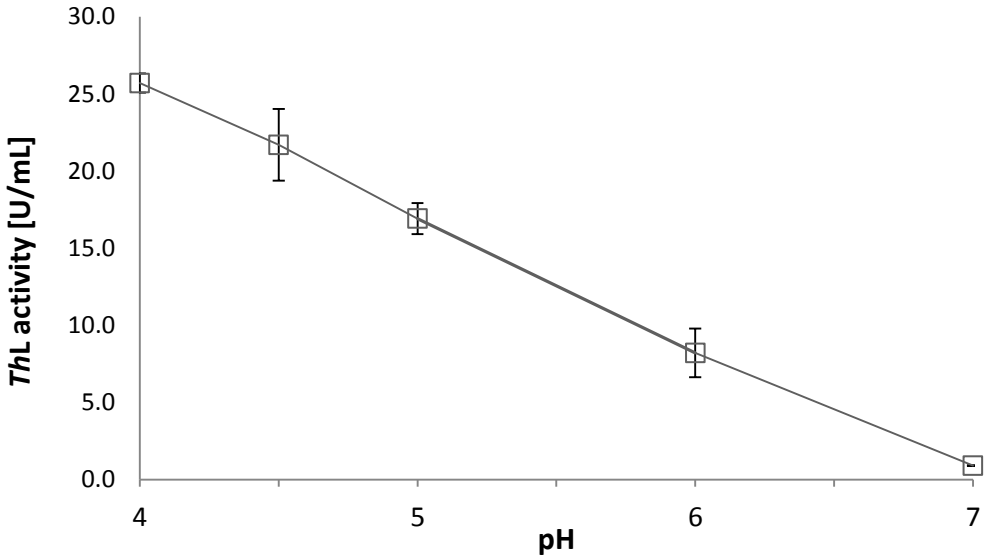


Figure 26: pH dependency of the activity of *Th*L [U/mL].

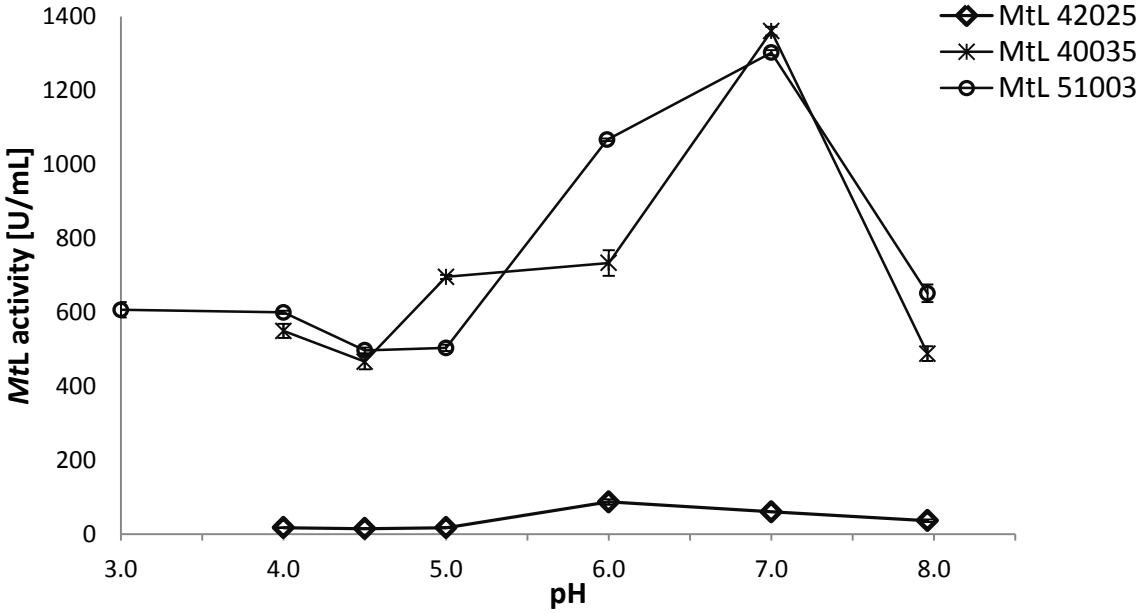


Figure 27: pH dependency of the activity of *Mt*L [U/mL]. The pH optimum profiles of the batches 40035 and 51003 are very similar; for batch 42025 significant lower laccase activities were determined.

3.1.4 Dependency of the laccase activity on the buffer conditions

Next to pH and temperature, the activity of an enzyme can be influenced by the presence of salts. It is known that laccases are inhibited by ions, such as fluoride, cyanide, hydroxides [31]. This inhibition is explained by the interruption of the internal electron transfer of the laccase due to the ions binding to the type 2 and type 3 copper sites [31]. To test the influence of the buffer ions on the activity of *Trametes hirsuta* laccase activity measurements were performed. Therefore the activities of *Th*L, diluted in two concentrations of sodium acetate buffer and ammonium acetate buffer, were compared. The results of the activity measurements can be found in figure 28. No significant dependency of the *Th*L activity on the type or concentration of the buffer could be found.

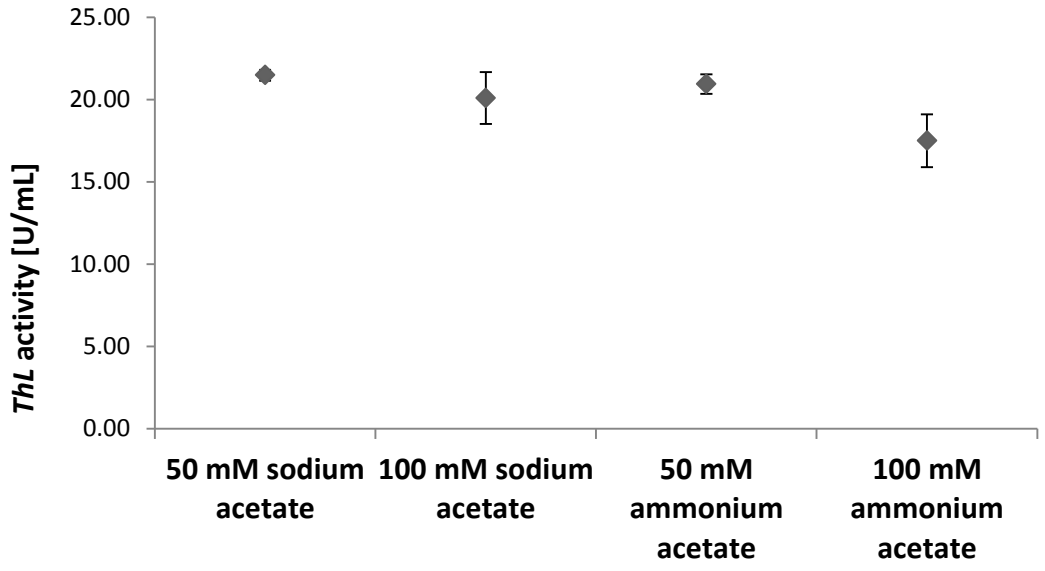


Figure 28: Dependency of the *Th*L activity on the buffer type and ionic strength. The sodium acetate buffer and ammonium acetate buffer provide a pH of 4.5.

3.1.5 SDS-PAGE analysis of the tested laccases

To estimate the molecular weight of *Mt*L, a sodium dodecyl sulfate gel electrophoresis was performed. This procedure is based on the denaturation of the enzyme and binding SDS to the amino-acid side chains, resulting in a uniform negative charged enzyme. The denaturized protein sample is then separated in an electric field, depending foremost on the molecular size of the enzyme and not on its conformation. After separation, the SDS-Page gel is stained with a Coomassie Blue solution to make the protein bands visual. The molecular size of the *Mt*L batches 42035, 51003, 40035 was determined to be 110 kDa, by comparing the bands to a standard. In previous project studies the molecular weight of *Th*L was determined to be 66 kDa. The stained SDS-Page gel can be found in figure 29.

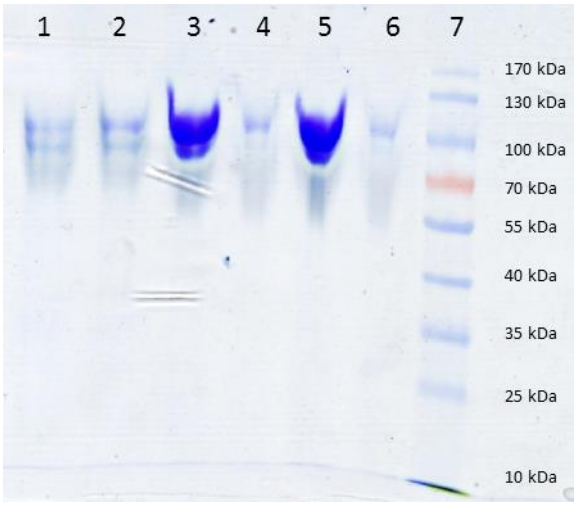


Figure 29: SDS-Page gel stained with Coomassie Blue to determine the molecular weight of (1) *Myceliophthora thermophila* Laccase 42035, (2) *Myceliophthora thermophila* Laccase 42035 1:2, (3) *Myceliophthora thermophila* Laccase 51003 1:10, (4) *Myceliophthora thermophila* Laccase 51003 1:50, (5) *Myceliophthora thermophila* Laccase 40035 1:10, (6) *Myceliophthora thermophila* Laccase 40035 1:50, by comparing the bands to a standard (7). The different protein concentrations of the three *Mt*L batches are visual in this figure. The band (1) of the not diluted *Mt*L 42035 is weaker than the bands (3) and (5) of the in water diluted *Mt*L 51003 and *Mt*L 40035.

3.2 Inhibition test of *Mt*L

In order to optimize the drying performance of the laccase mediator system, it is important to determine possible laccase inhibitors present in the long alkyd resin. The long alkyd resin contains the chelating agent DMEA in a concentration of 0.08 % w/w. To determine the effect of DMEA on the catalytic activity of *Mt*L, the enzyme was stored in a 0.08 % DMEA buffer solution. The measured laccase activities compared to a control can be found in figure 30. Over a storage time of 5 days at a constant temperature of 25°C, the enzyme loses about 44 % of its activity, independent of the presence of DMEA. All in all, a possible inhibiting effect of DMEA on the activity of *Mt*L was not determined by this test.

In the further course of this study, the storage stability of the laccase and mediator in the formulated long alkyd resin was investigated by oxygen consumption measurements (chapter 3.5.).

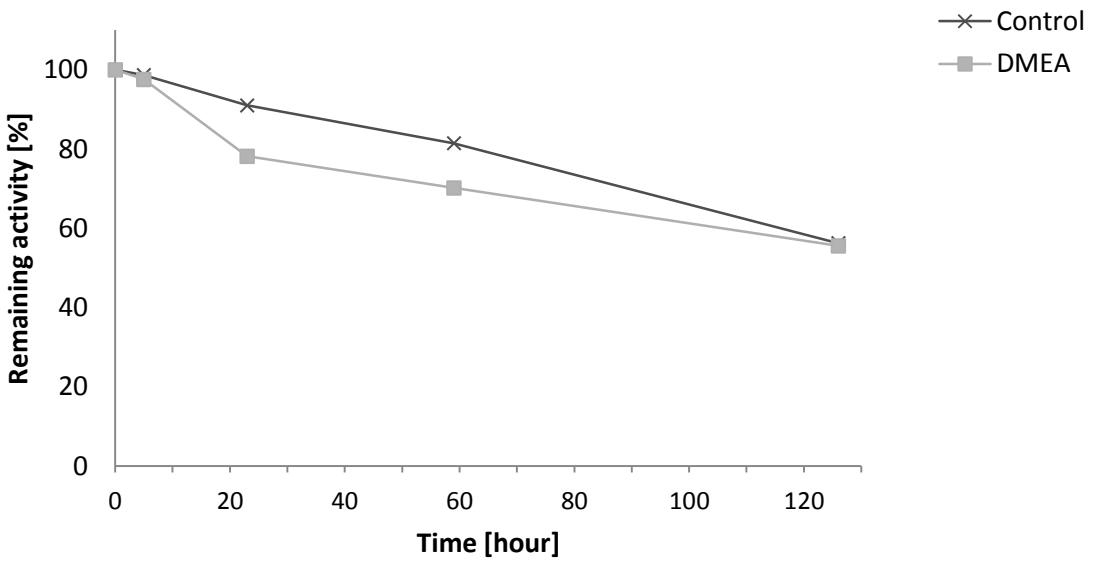


Figure 30: Changes of the *Mt*L activity during storage at 25°C in a 0.08 % DMEA buffer solution (100 mM sodium phosphate buffer, pH7) compared to the control.

3.3 Oxygen consumption measurement

Oxygen consumption measurements allow it to follow and evaluate the cross-linking reaction, as the amount of consumed oxygen is a parameter of the quality of the oxidation reaction and laccase activity. In this work, oxygen consumption measurements are used to study the pH dependency of the cross-linking reaction to test various substrates and to screen for efficient laccase mediators. The advantages of this method are the need of low amounts of substrate and enzyme, as well as fast and easy execution. Further, it could be seen, that the results of the measurements are in good agreement with drying time recorder measurements and FTIR analysis (chapter 3.4 and 3.6.).

Oxygen consumption measurements were performed in aqueous media. The experimental set-up is divided in two steps, and is described in chapter 2.3. In a first step, oxygen is consumed until the mediator is fully oxidized by the laccase and the measured oxygen concentration reaches a stable value again. Then, the cross-linking reaction is started by adding the substrate, resulting in a second decrease of the oxygen concentration. At this step oxygen is consumed during the oxidation of the unsaturated fatty acid side chains of the substrate. The second decrease of oxygen is used to evaluate the quality of the cross-linking reactions and is given in the following figures of this chapter. The consumption of oxygen was measured in duplicates, and the stated oxygen consumption has been calculated as an average value of these repetitions.

3.3.1 Inactive enzyme

As a negative control, oxygen consumption measurements were performed using inactive *Th*L and *Mt*L. To a 0.1 mM ABTS buffer solution containing inactive enzyme, long alkyd resin was added as a

substrate. The oxygen consumption measured using inactive *Th*L and *Mt*L is compared to an active enzyme shown in figure 31 and figure 32. For both inactive laccases an initial oxygen concentration decrease of 10 % was determined. This is caused by diffusion changes due to adding the rather viscous substrate to the aqueous media because oxygen concentration recovers within the first 25 minutes and reaches a stable value again. Further decrease of the oxygen concentration was not observed, indicating that no cross-linking reaction of the substrate occurred. These results proof that only active laccase is able to oxidize the mediator and catalyze the cross-linking of long alkyd resin resulting in oxygen consumption.

3.3.2 pH dependency of the crosslinking reaction

The pH dependency of the LMS catalyzed cross-linking of the long alkyd resin is studied by oxygen consumption measurements at different pH conditions. The measurements were performed in a 0.1 mM ABTS buffer solution, with an enzyme activity of 0.03 U/mL in the total reaction volume. The oxidation reaction was started by adding the substrate long alkyd resin.

The oxygen consumption measured for *Th*L at various pH conditions is illustrated in figure 31. After the addition of substrate, a fast consumption of oxygen could be observed for all tested pH conditions and oxygen consumption reaches a maximum within the first 20 minutes. The fast consumption of oxygen can be explained by a fast hydroperoxide formation at the beginning of the reaction. This is followed by hydroperoxide decomposition and radical chain propagation; at this stage oxygen consumption decreases slowly until the end of the measurement. The highest amount of oxygen consumed was determined at pH 6 reaching 90 % oxygen consumption after about 20 minutes. The least amount of oxygen consumption was measured at pH 4 with 65 % oxygen consumption in the first 20 minutes.

Similar to the oxygen consumption with *Th*L, the consumption for *Mt*L at pH 6, 5 and 4.5 is fast at the beginning, goes through a maximum, and then slowly decreases. At pH 7 and 8 oxygen consumption is similar fast at the beginning, and then keeps increasing in a lower rate. For *Mt*L the highest oxygen consumption of about 80 % was determined at pH 7 after about 900 minutes. At pH values below 7, oxygen consumption reaches a maximum in the range of 75 % to 60 % within the first 25 minutes, but then oxygen consumption decreases very fast reaching more stable oxygen consumption rates below 50 %. Overall lower amounts of oxygen are consumed at pH values below 7. The stated oxygen consumptions are calculated average values of two measurements and are plotted against time in figure 32.

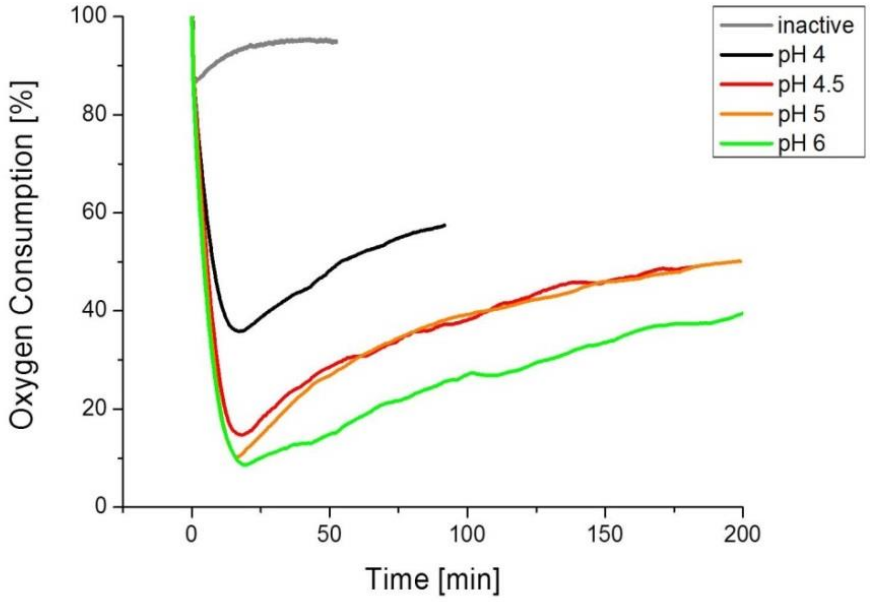


Figure 31: Oxygen consumption versus time measured for *Th*L (0.03 U/mL) and ABTS (0.1 mM) at various pH conditions after adding long alkyd resin. The consumed oxygen is compared to the measurements performed with inactive *Th*L.

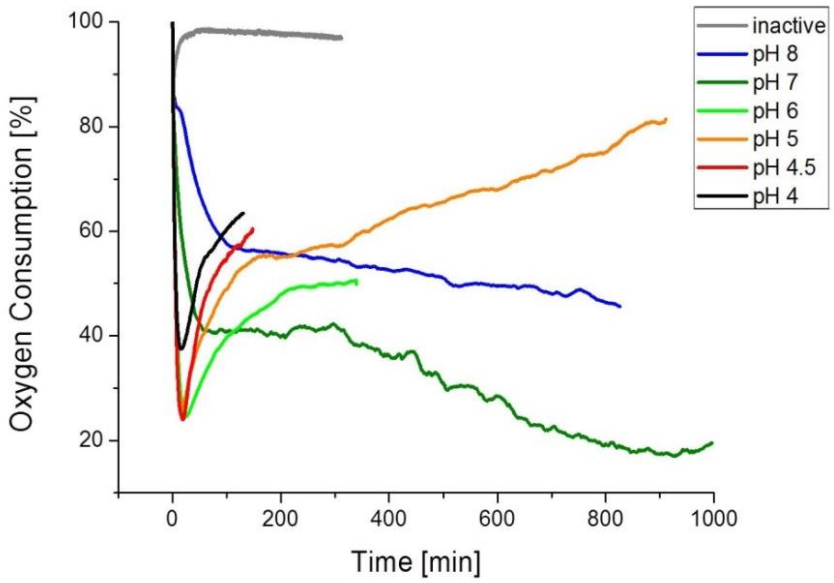


Figure 32: Oxygen consumption measured for *Mt*L (0.03 U/mL) and ABTS (0.1 mM) after the addition of long alkyd resin at various pH conditions. The consumed oxygen is compared to the measurements performed with inactive *Mt*L at pH 7.

3.3.3 Substrate Screening

Alkyd resins have a diverse composition of oils in their formulation depending on the final application, such as linseed oil, tall oil, soy oil or sunflower oil. In order to determine the specificity of *Th*L and *Mt*L towards different fatty acid components in alkyd resins, oxygen consumption measurements were performed. The fatty acid compositions of the tested linseed oil, long alkyd resin and castor oil based alkyd resin, are listed in table 2. The oxygen consumption plotted against time measured for various substrates can be found in figure 33 and figure 34.

Tributyrin emulsion

As a negative control tributyrin, a saturated triglyceride was tested as a laccase substrate. Due to the lack of double bonds an oxidation of the substrate is not possible, which was confirmed by the lack of oxygen consumption. The obtained oxygen consumption rate is very similar to the performed measurements with inactive enzyme, as it can be seen in figure 34.

Linseed oil emulsion

Linseed oil has as major constituent linolenic acid, whereas for sunflower oil and soya oil it is linoleic acid [9]. Oxygen consumption measurements, using a linseed oil emulsion as substrate were performed with both laccases. Oxygen consumptions measured for *Th*L are indicated in figure 33; oxygen consumptions measured for *Mt*L are shown in figure 34. For both enzymes, a significant higher amount of oxygen is consumed using the linseed oil emulsion as a substrate compared to long alkyd resin. The higher oxygen consumption can be explained by a higher fatty acid percentage in the linseed oil emulsion. The tested linseed oil emulsion has a fatty acid percentage of 62 %, long alkyd resin a 42 % percentage. Additionally, the laccase might be inhibited by ingredients of the long alkyd resin that are not present in the linseed oil emulsion. The different oxygen consumption rates can also be caused by structural differences of the linseed oil and the synthetic long alkyd resin. Due to structural differences the LMS catalyzed oxidation of the fatty acid side chains in long alkyd resin might be hindered compared to linseed oil.

Linoleic acid emulsion

To test the oxidation ability of the laccase on a free fatty acid, linoleic acid was tested as a substrate. The oxygen consumption measured with *Mt*L (0.03 U/mL) in a 0.1 mM ABTS buffer solution (pH 7) can be found in figure 34. For the linoleic acid emulsion less oxygen was consumed than for the linseed oil emulsion. Linseed oil is a triglyceride with linolenic acid (18:3) as main constituent, next to linoleic acid (18:2), oleic acid (C18:1) and saturated fatty acids. As mentioned earlier, the cross-linking and polymerization in drying oils takes place at the unsaturated centers or double bonds of the fatty acids [66]. Linoleic acid is a polyunsaturated fatty acid with two double bonds; linolenic acid with the same chain length as linoleic acid has three double bonds. Because of the additional double bond linolenic acid has a higher potential to be oxidized than linoleic acid, which results in a higher oxygen consumption for linseed oil emulsion. Further, the variance of fatty acid side chains in linseed oil might benefit the oxidative cross-linking reaction.

Methyl linoleate emulsion

Many studies use methyl or ethyl esters of fatty acids as model compounds to study the mechanism of the drying of alkyd resin paints, as they can be easier analyzed by standard analysis techniques [67]. Methyl linoleate and linoleic acid differ in their chemical structure only by the methyl group. For

the methyl linoleate emulsion significant less oxygen was consumed than for the linoleic acid emulsion most likely due to the lack of the carboxylic group.

Trilinolein emulsion

Oxygen consumption measurements with trilinolein, a triglyceride with solely linoleic acid as constituent, were performed. For the trilinolein emulsion less oxygen was consumed than for the free linoleic acid emulsion, indicating that the oxidation reaction is somehow hindered. Reasons for that might be emulsion differences or geometric differences between the trilinolein and free fatty acid emulsion.

Castor oil based alkyd resin

In previous studies it was claimed that conjugated fatty acids undergo a different oxidation mechanism than non-conjugated unsaturated fatty acids. The relatively low bond dissociation energy of the bis-allylic hydrogen in non-conjugated polyunsaturated fatty acids makes it highly susceptible for autoxidation. The hydrogen can be abstracted easily, initiating a radical chain reaction and autoxidation [9]. This could also be observed by oxygen consumption measurements, where less oxygen for the castor oil based alkyd resin was consumed than for the linseed oil based long alkyd resin, which is shown in figure 34. The only difference in their composition is the conjugation of their main fatty acid constituent linoleic acid. Castor oil based alkyd resin has mainly conjugated linoleic acid and long alkyd resin mainly non-conjugated linoleic acid as constituents. The differences of the oxygen consumption are mainly caused by different oxidation mechanisms of conjugated and non-conjugated fatty acids.

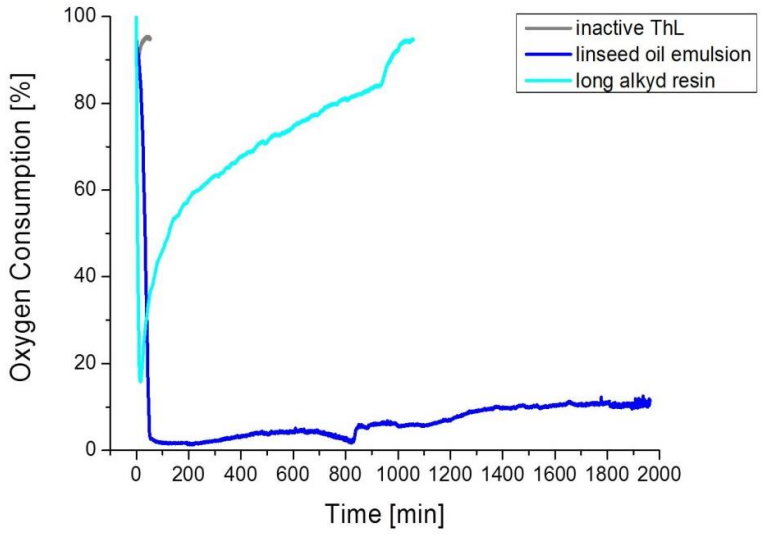


Figure 33: Oxygen consumption for *Th*L (0.03 U/mL) with linseed oil emulsion and long alkyd resin as substrate, in a 0.1 mM ABTS buffer solution (pH 4.5). As a negative control the oxygen consumptions are compared to the oxygen consumed with inactive *Th*L.

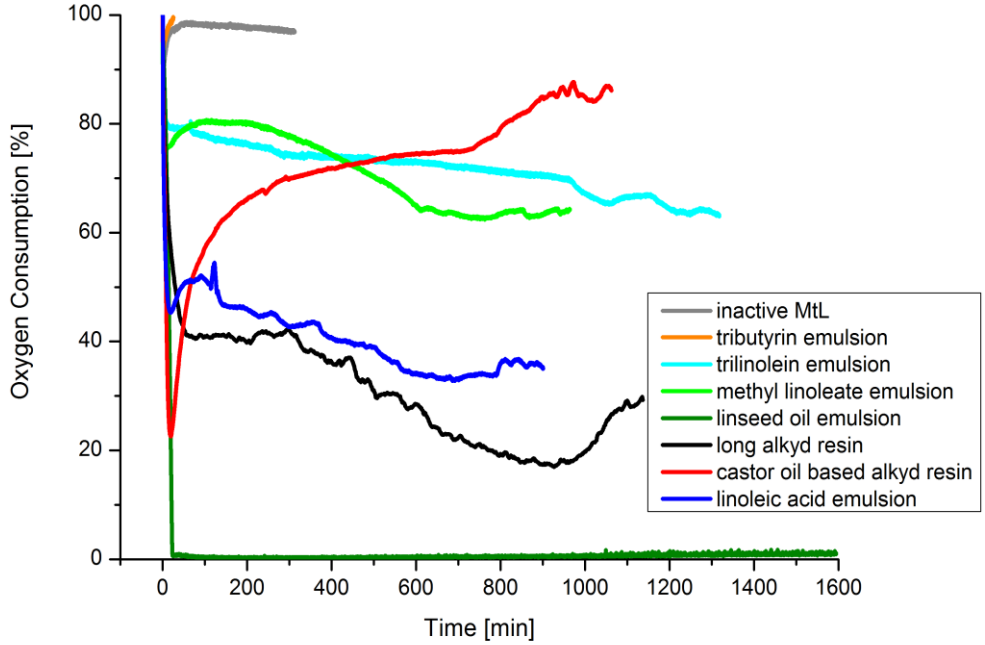


Figure 34: Oxygen consumption versus time comparing various substrates treated with *Mt*L (0.03 U/mL) and ABTS (0.1 mM). As a negative control the oxygen consumptions are compared to the oxygen consumed with inactive *Mt*L.

Cross-linking with higher concentrations of ABTS and MtL

The effect of increasing the mediator and laccase concentration on the oxygen consumption was studied using long alkyd resin, linseed oil emulsion, methyl linoleate emulsion, trilinolein emulsion, and castor oil based alkyd resin as substrates. Therefore the concentration of ABTS was increased from 0.1 mM to 1 mM in the buffer solution and the concentration of *Mt*L from 0.03 U/mL to 0.05 U/mL. For the methyl linoleate emulsion increasing the ABTS and laccase concentration resulted only in a slight increase of oxygen consumption. For the trilinolein emulsion using mediator and laccase in a higher concentration resulted in a significant increase of oxygen consumption of about 60 % after 800 minutes. For castor oil based alkyd resin an increase of the mediator and laccase concentration resulted in an increase of the oxygen consumption of about 20 % after 800 minutes. As for linseed oil already a maximum of 98 % oxygen consumption within the first 25 minutes was reached with the lower mediator and laccase concentrations, no significant increase could be observed using higher concentrations of mediator and laccase. The results of the oxygen consumption measurements with increased mediator and laccase concentrations for long alkyd resin, methyl linoleate emulsion, trilinolein emulsion, castor oil based alkyd resin, and linseed oil emulsion are shown in figure 35. The cross-linked long alkyd resin and linseed oil were further analyzed by gas chromatography (chapter 3.7.2) and ATR-FTIR analysis (chapter 3.7.1)

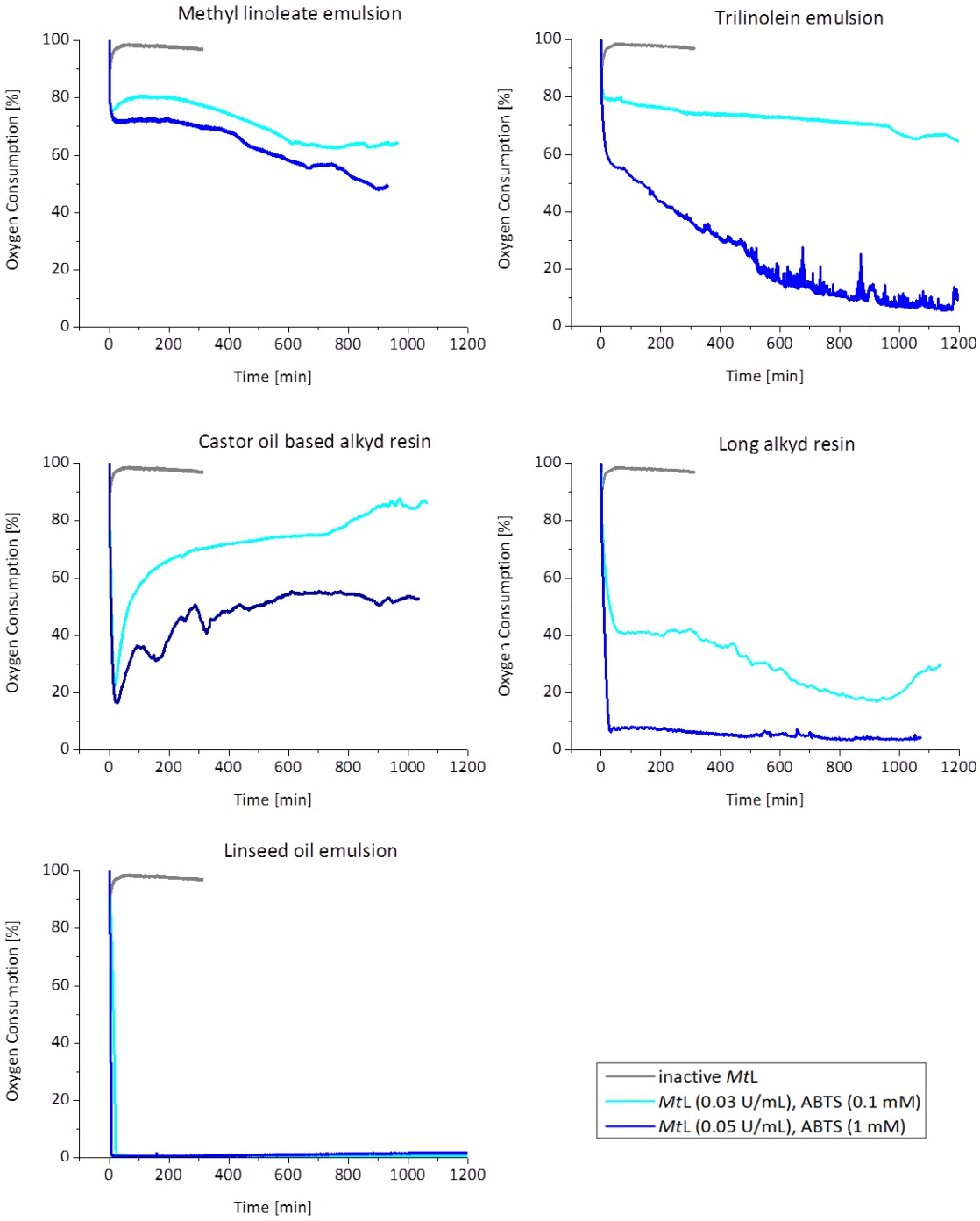


Figure 35: Oxygen consumption of the methyl linoleate emulsion, trilinolein emulsion, castor oil based alkyd resin, long alkyd resin and linseed oil emulsion. The measurements with increased mediator and laccase concentration (1 mM ABTS and 0.05 U/mL *Mt*L) (dark blue) are compared the measurements performed with lower concentrations (0.1 mM ABTS and 0.03 U/mL *Mt*L) (light blue).

3.3.4 Screening for laccase mediators

It could be shown in previous studies that a laccase mediator system catalyzes the drying of long alkyd resin, using *Trametes hirsuta* laccase and ABTS or HBT as mediator [1]. Because of their relatively high costs and potential toxicity it is important to find alternative laccase mediators to the synthetic mediators ABTS and HBT [68]. Many papers concentrate on the search of natural compounds as laccase mediators and the tested compounds have been previously described as laccase enhancers [27][30][38].

The search for alternative *Mt*L mediators was started by measuring the oxygen consumption in aqueous media and comparing various potential laccase enhancers. Oxygen consumption measurements are ideal as a first screening method for laccase mediators, with the advantage of low substrate requirement and fast execution. Via oxygen consumption measurements efficient laccase mediators were identified, followed by further testing via FTIR analysis and drying recorder tests.

The oxygen consumption during the oxidative drying of long alkyd resin using different laccase mediators can be found in figure 36. All mediators are present in a concentration of 0.1 mM in buffer and the measurements were performed with an *Mt*L activity of 0.2 U/mL in the total reaction volume. As a negative control, inactive *Mt*L and active *Mt*L without mediator were used. As expected, no oxygen was consumed using inactive *Mt*L. For active *Mt*L without mediator a slight oxygen concentration decrease of 10 % was observed, which can be explained the low potential of the laccase to directly oxidize the substrate.

Judging by the very low oxygen consumption measured for vanillic acid, it is not a suitable laccase mediator to catalyze the oxidation of long alkyd resin. For the measurements with vanillic acid, a very similar oxygen consumption rate as for the negative control without mediator was determined. Because of the low oxygen consumption, it can be concluded that no cross-linking of the long alkyd resin occurred. For vanillin, syringaldehyde, ethyl vanillin, acetosyringone and acetovanillone oxygen consumption was clearly observed over time. Their oxygen consumption profiles indicate an oxidation of long alkyd resin, confirming their potential as alternative laccase mediators. Acetovanillone shows a particular promising oxygen consumption rate, being similar to the oxygen uptake rate measured for ABTS.

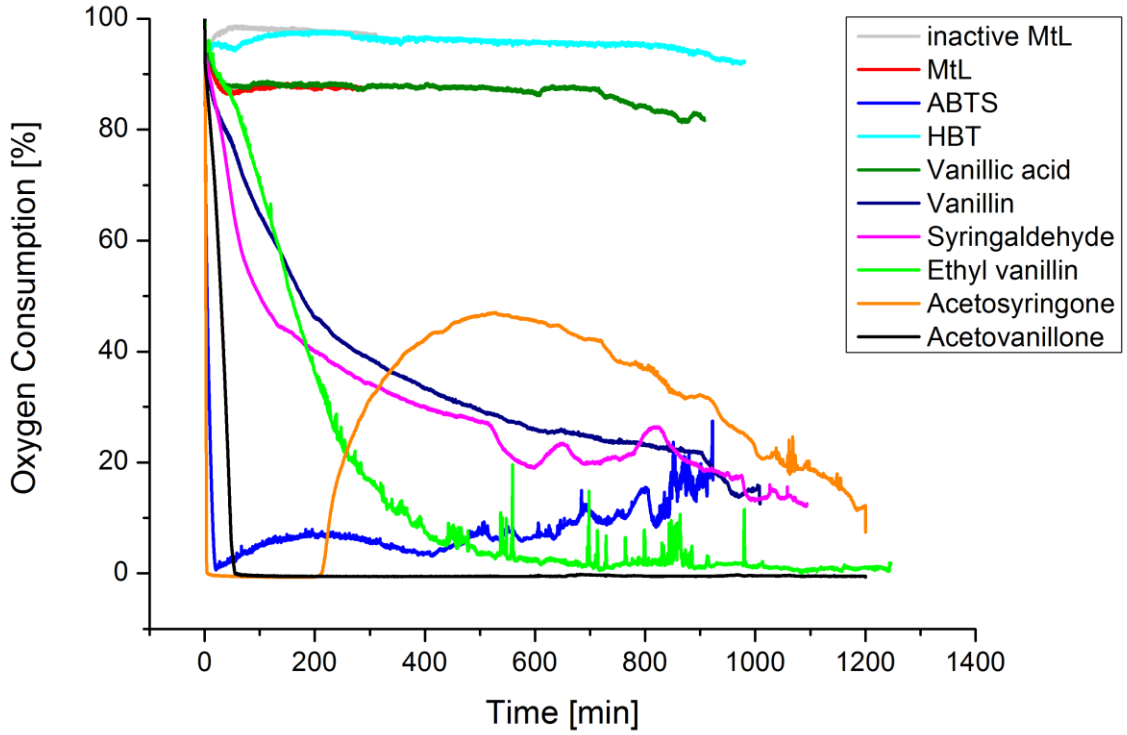


Figure 36: Oxygen consumption versus time measured for different laccase mediators during the cross-linking of long alkyd resin. The mediators are present in a concentration of 0.1 mM in the buffer solution (pH 7). The measurements were performed using an *Mt*L activity of 0.2 U/mL in the total reaction volume.

3.4 Testing the drying performance

The tested long alkyd resin is mainly a chemical drying binder, and obtains only minor drying reactions without catalyzer. Drying time recorder analysis is a well-known tool in coating industry to study the drying process of alkyd resin films. In this study drying time recorder analysis was used to screen for potential laccase mediators and to investigate the storage stability of the laccase and the mediator in the long alkyd resin.

3.4.1 Screening for laccase mediators

Next to oxygen consumption measurements and FTIR analysis, the drying ability of potential laccase enhancers was studied using drying time recorder measurements. The various stages of drying observed in drying long alkyd resin films are shown in figure 37.

As a positive control, long alkyd resin was treated with a cobalt siccative. The known laccase mediator ABTS was tested in different concentrations, in order to determine the required concentration for the drying of long alkyd resin. A low mediator concentration of 0.1 mM and an activity of 44 U/mL *Mt*L are not sufficient to dry the long alkyd resin. To reach the dry stage a concentration of 1 mM ABTS and 44 U/mL *Mt*L is needed. Increasing the mediator concentration

from 1 mM to 2 mM ABTS and keeping the laccase concentration at the same value, doesn't have a big effect on the drying result. This was also observed using vanillin and acetovanillone, where only small improvements of the drying performance could be detected when increasing the mediator concentrations.

To compare the catalytic ability of the potential laccase enhancers a concentration of 2 mM mediator and 44 U/mL laccase in the drying long alkyd resin film was chosen. In general, all mediators that were tested positive for oxygen consumption also reach a drying stage. For vanillic acid no oxygen consumption was measured and also no drying was observed. Ethyl vanillin showed a bad drying performance, with a ripped film until the end of the measurements. Vanillin as laccase mediator showed better a drying performance, reaching the basic trace stage after 36 h. The best drying performances were overserved for acetovanillone, acetosyringone and syringaldehyde with equally good drying results. By increasing the vanillin concentration from 2 mM to 4 mM with a stable laccase concentration, the drying performance was improved to the level of acetovanillone, acetosyringone and syringaldehyde. To sum up, the drying ability of acetovanillone, acetosyringone, syringaldehyde (in a concentration of 2 mM and 44 U/mL *Mt*L) and vanillin (4 mM and 44 U/ml *Mt*L) is comparable to the drying results with ABTS (2 mM, 44 U/mL *MtL*). However, it has to be mentioned that drying time recorder measurements show low sensitivity and small differences in the drying ability are not detectable.

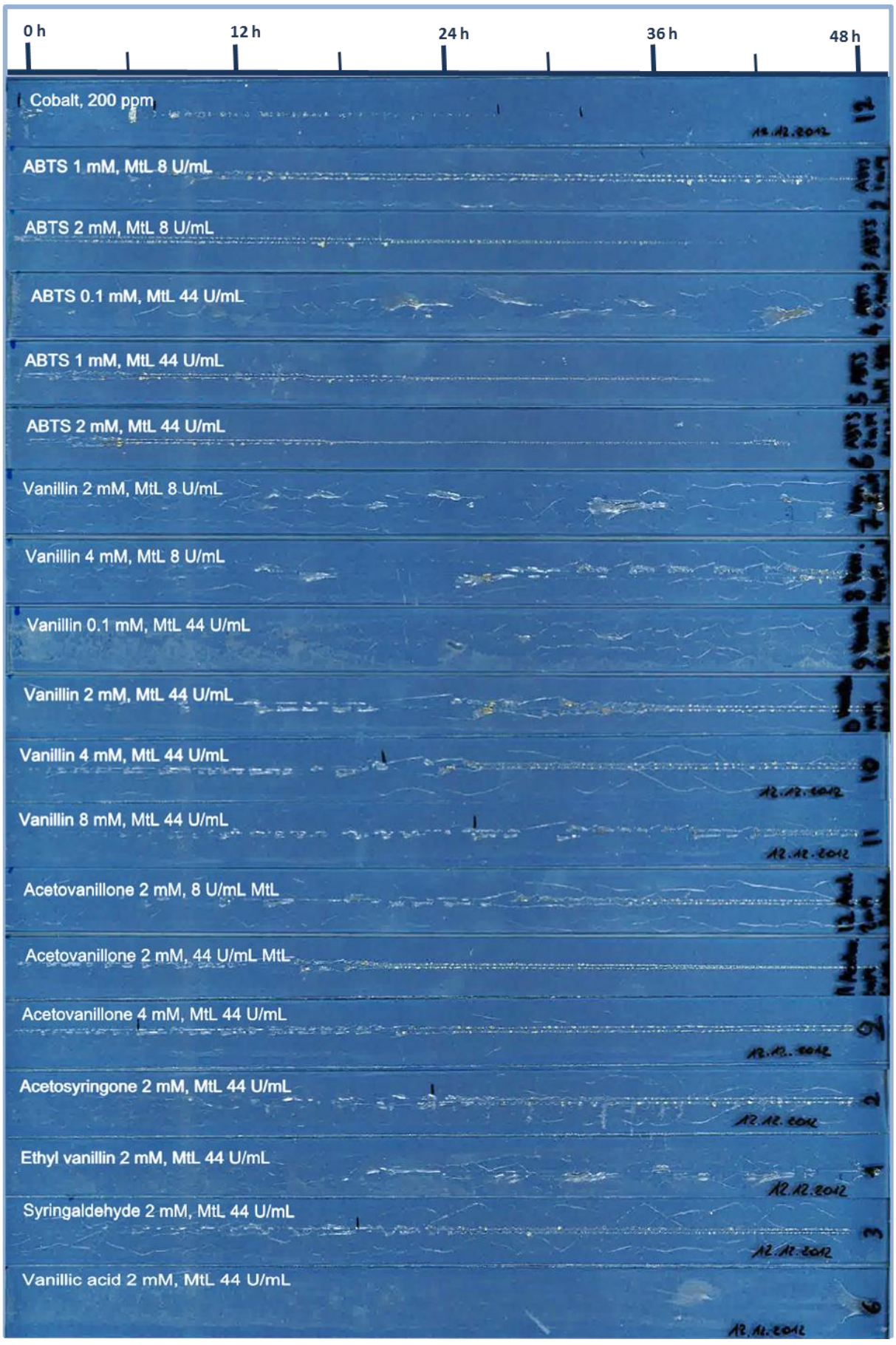


Figure 37: Drying time recorder measurements performed with various types of mediators, varying the concentration of mediator and laccase. Long alkyd resin was applied with a film thickness of 152 µm.

3.5 Storage stability test

Coatings of alkyd resins contain many different chemical compounds including the catalyst, that all need to be stable over the time of storage. In our case, it could be observed that the drying performance clearly decreased after storage of the laccase and mediator in the long alkyd resin. To investigate the storage stability of the laccase and mediator in the long alkyd resin coating, drying time recorder and oxygen consumption measurements were performed.

3.5.1 Drying time recorder measurement

Drying time recorder tests showed a strong decrease of the drying performance in dependence of the storage time (figure 38). ABTS/HBT and *Mt*L were added to the long alkyd resin three days before testing the drying performance; in this case the dry stage was reached after 33 hours. A slower drying of the alkyd resin film was observed by storing the mediators and the laccase for 5 days before performing the drying time recorder test. After the storage of *Mt*L and ABTS/HBT for 25 days in the long alkyd resin the dry stage was not reached in the alkyd resin film and the needle ripped through the film.

ABTS/HBT was stored for 25 days in the long alkyd resin, without laccase present to test the effect of storage on the mediators. The missing laccase component was added 24 h before the drying recorder test. For the mediators ABTS/HBT the storage of 25 days in long alkyd resin had no significant influence on the results of the drying recorder tests. This was in contrast to the drying performance of the sample with laccase that was stored in long alkyd resin, where a clear decrease of the drying performance was observed, indicating an activity loss of the laccase. In this case the laccase was stored in the long alkyd resin for 25 days and ABTS/HBT was added 24 h before the drying recorder test. The drying recorder test showed for *Mt*L (with a storage time of 25 days) a ripped alkyd resin film until the end of the measurement, compared to ABTS/HBT (25 days), where the dry stage is reached after 28 h. For the joint storage of laccase and mediators (25 days) or storage of solely *Mt*L (25 days) no drying of the long alkyd resin was observed within 48 h. This proofs that the laccase activity is highly affected by the storage in long alkyd resin.

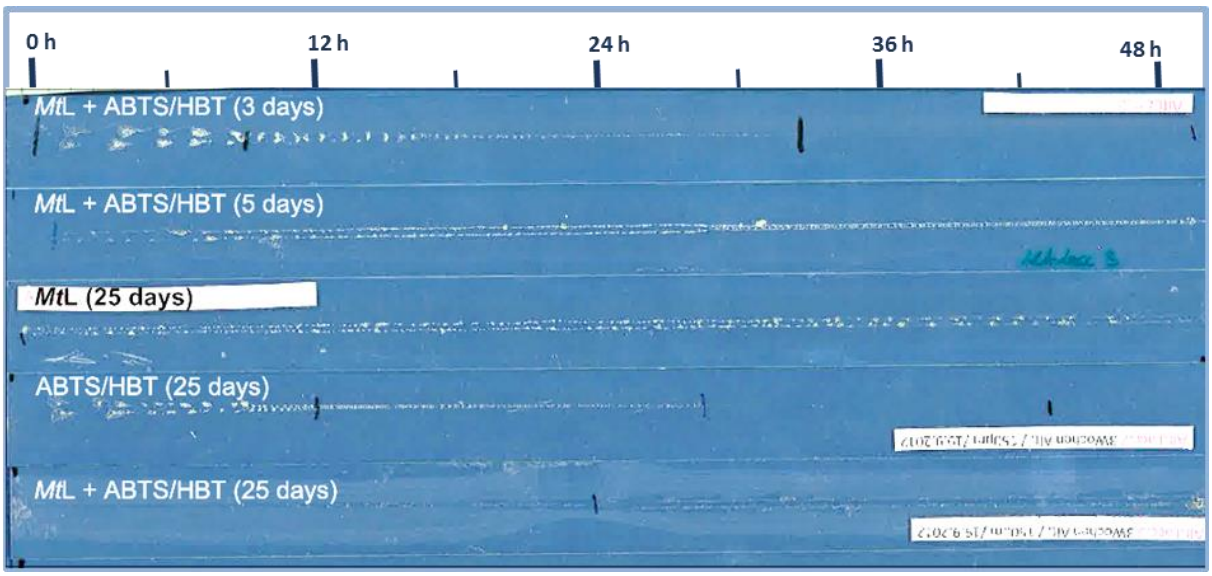


Figure 38: Drying time recorder measurements after 3, 5 and 25 days of joint storage of laccase and mediators – *Mt*L + ABTS/HBT (3 days), *Mt*L + ABTS/HBT (5 days) and *Mt*L + ABTS/HBT (25 days) - in the long alkyd resin. The drying results are compared to separate stored laccase - *Mt*L (25 days) - and mediator - ABTS/HBT (25 days) – in long alkyd resin. In this case, the missing component was added 24 h before the measurement. The film thickness applied on the glass stripe was 152 µm.

3.5.2 Oxygen consumption measurement

The storage effect on the laccase activity was further tested by oxygen consumption measurements. Therefore stored laccase in long alkyd resin was added to a buffer solution, and the cross-linking reaction was started by adding a mediator. The measured oxygen consumption can be associated to the activity of the laccase. A loss of laccase activity during storage results in lower amounts of oxygen consumed. No significant difference between the storage of long alkyd resin in glass or polypropylene vessels could be observed by these measurements. In both cases the activity of laccase decreases clearly after a storage time of 25 days in the long alkyd resin, visual in a more than 20 % lower oxygen consumption. After 10 and 17 days no significant decrease of oxygen consumption could be observed.

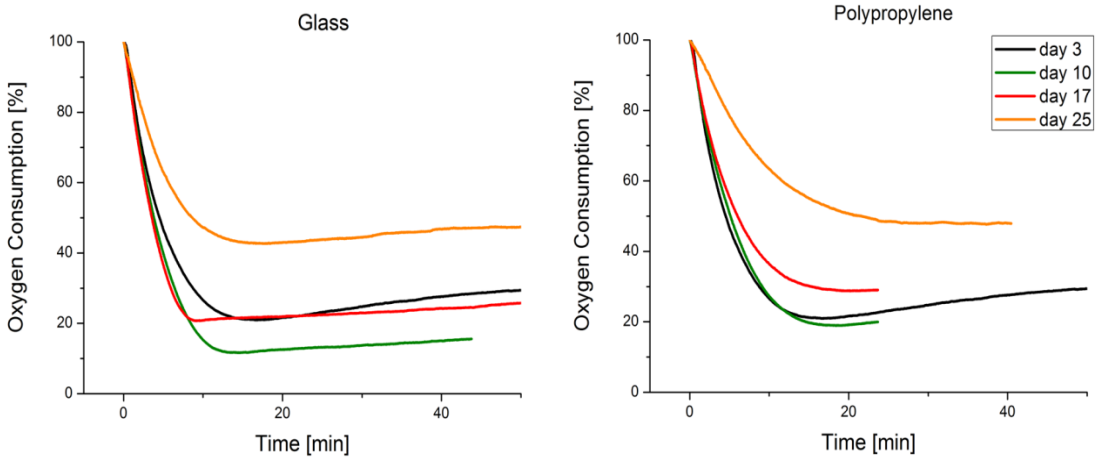


Figure 39: Oxygen consumption measured after storage of laccase in long alkyd resin for 3, 10, 17 and 25 days using a glass or a polypropylene storage vessel.

3.6 Process monitoring via Fourier transform infrared spectroscopy

FTIR analysis is a well-known method to follow the drying of alkyd resins and model compounds on a molecular level [19][69][70]. During the oxidation of alkyd resin IR absorptions go through transition, which can be associated to the oxidation reaction of alkyd resin. The decrease of the *cis* **H-C**=CH symmetric stretching vibration at 3010 cm^{-1} is especially useful to follow the disappearance of the substrate in time; as it can be associated to the first step of the cross-linking reaction. In this step a hydrogen is abstracted at the activated methylene group, followed by a rearrangement of the resulting radicals and disappearance of the *cis* **H-C**=CH vibration [67]. In prior project related studies it was shown that both cobalt catalysts and the LMS significantly decrease the peak at 3010 cm^{-1} and increase the band in the region 3100-3600 cm^{-1} which is assigned to OH-containing compounds (e.g. hydroperoxides, alcohol and acids) [1]. In this study FTIR analysis is used to screen for efficient laccase mediators and to study the cross-linked linseed oil and long alkyd resin formed by the LMS catalyzed drying reaction.

Figure 40 shows the ATR-FTIR spectra (1400-3750 cm^{-1}) recorded during the LMS catalyzed oxidation of long alkyd resin over a reaction time of 168 h. The FTIR spectra of the negative control, long alkyd resin that was treated solely with *Mt*L, are shown in figure 41.

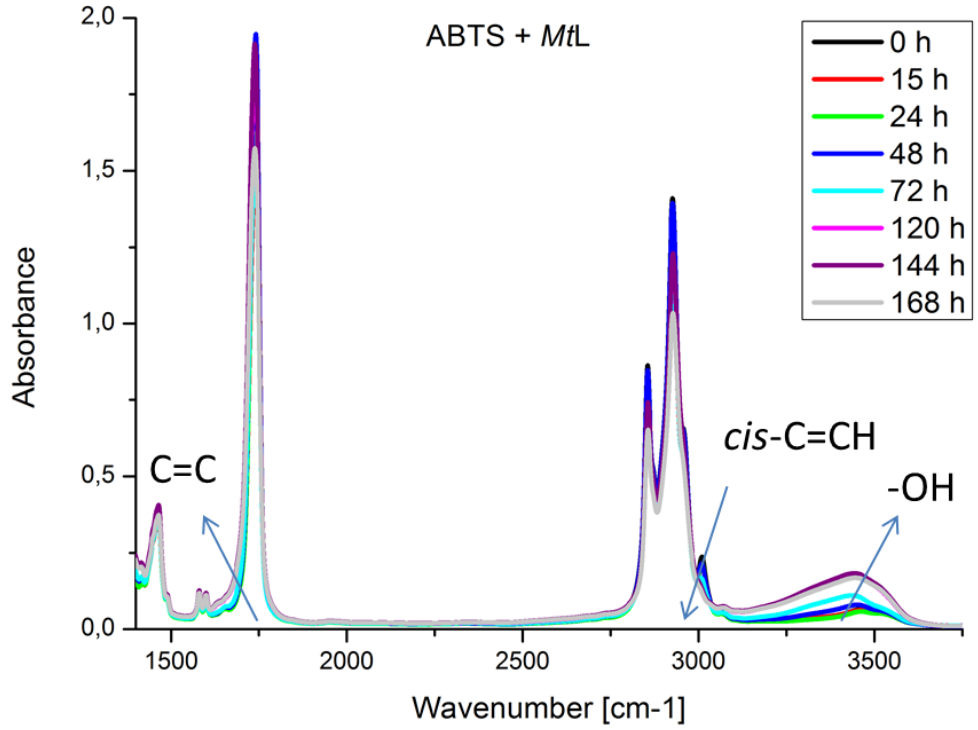


Figure 40: ATR-FTIR spectra (1400-3750 cm^{-1}) during the LMS catalyzed oxidation of long alkyd resin. Long alkyd resin (pH 7) was treated with 2 mM ABTS and 44 U/mL *Mt*L and applied on a glass slide in a film thickness of 156 µm, after a 24 h incubation time. The arrows indicate the time increase. The peak at 3010 cm^{-1} decreases clearly during the catalyzed oxidation of long alkyd resin and the decrease can be associated directly to the first step of the oxidation process.

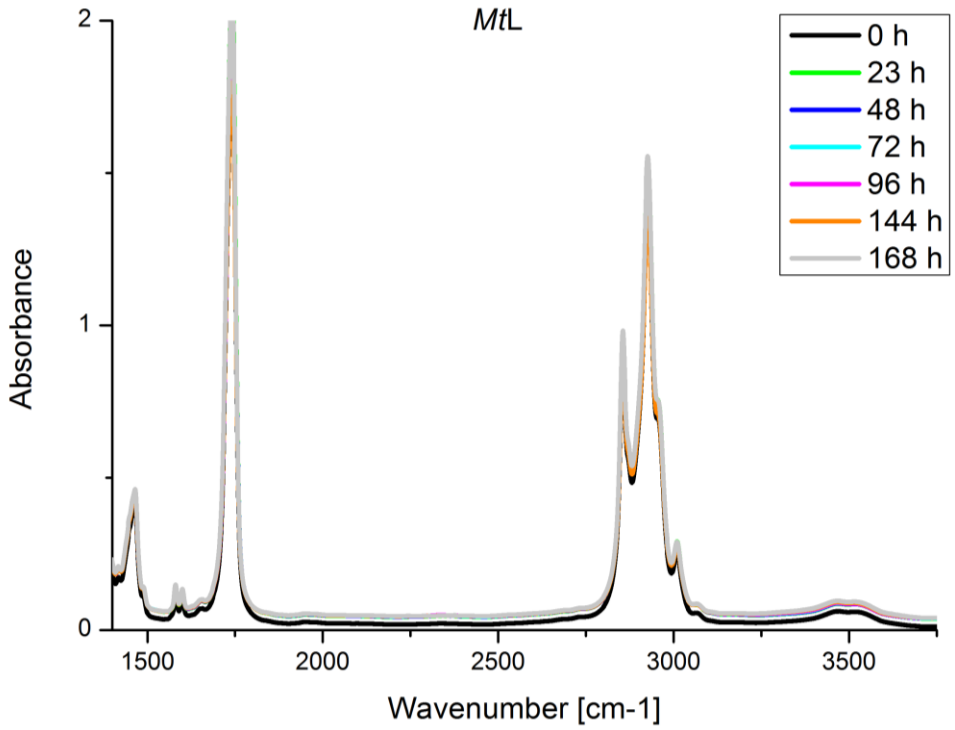


Figure 41: ATR-FTIR spectra (1400-3750 cm^{-1}) of long alkyd resin during an observation period of 168 h under the influence of solely *Mt*L. Because of the lack of mediator no oxidation related changes of the IR spectra were observed.

In contrast to LMS treated long alkyd resin, no significant changes of the IR absorptions were observed without adding a mediator to the reaction mixture. In the presence of the LMS, the *cis*-C=CH assigned peak at 3010 cm^{-1} decreased clearly over the time of the measurement. At the end of the observation period (168 h) only a small peak at 3010 cm^{-1} is left for the LMS treated long alkyd resin. Simultaneously the peak at 1720 cm^{-1}, corresponding to C=O, broadens during the oxidation reaction. In addition, the range of 3100-3600 cm^{-1} broadens, indicating the formation of hydroperoxides, alcohols and acids. These observed changes are in agreement with oxidation mechanisms generally found during the drying of alkyd resin.

3.6.1 Screening for laccase mediators

The activity of the laccase mediator system towards alkyd resin depends highly on the stability and reactivity of the mediator and the radical formed by mediator oxidation [71]. FTIR analysis serves us, next to oxygen consumption measurements and DTR measurements, as a third tool to find efficient laccase mediators. This method allows us to follow the oxidation of alkyd resin on a molecular level and get a better understanding of the drying potential of the laccase mediator system. As mentioned before the disappearance of the peak at 3010 cm^{-1} can be associated directly to the first step of the oxidation reaction of alkyd resin [67]. That is why the drying ability of different laccase mediators can be judged by the decrease of the double-bond assigned peak at 3010 cm^{-1} over time.

In this study FTIR analysis of drying long alkyd resin films using different mediators was performed and the changes of the peak assigned to the double-bond were observed over time. The peak area of each spectrum was integrated between 2992 and 2018 cm^{-1}. The ratio between the peak area at the time of the measurement and the area at the beginning was calculated for each mediator. The logarithmic plot of the data versus time is given in figure 42. The drying long alkyd resin films were treated with 2 mM mediator and 44 U/mL laccase. Additional FTIR analysis of long alkyd resin treated with varying concentrations of the mediators ABTS, vanillin, or acetovanillone and varying activities of *Mt*L were performed; these results can be found in the appendix (figure 54, figure 55 and figure 56).

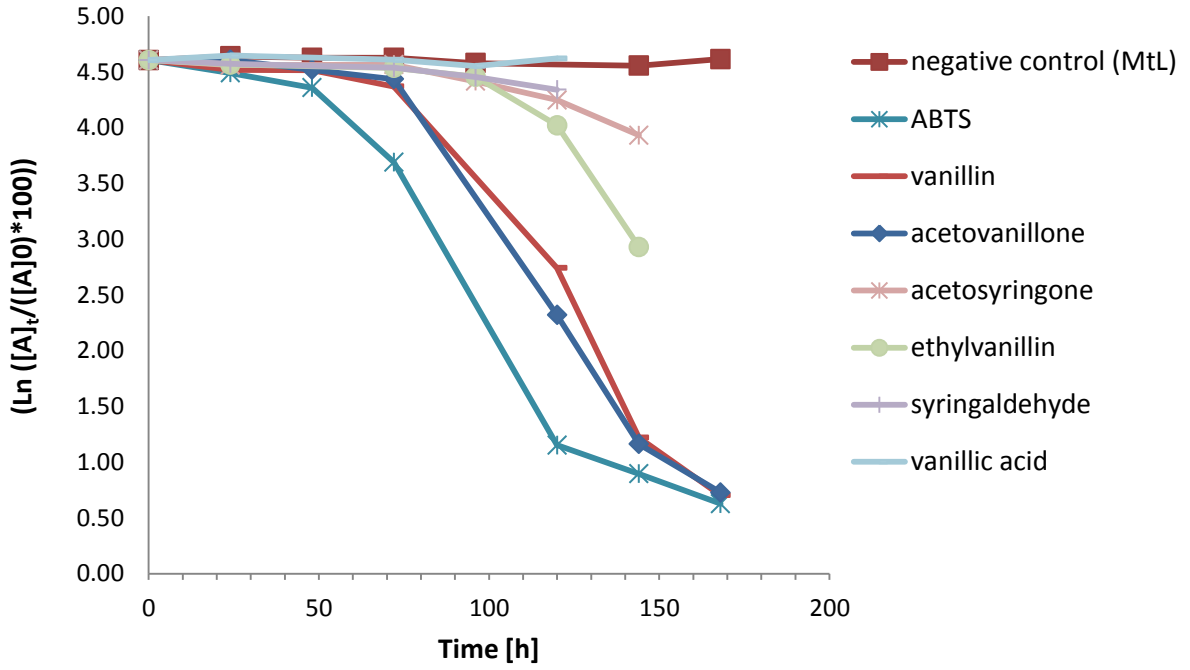


Figure 42: Changes of the *cis*-C=C-H symmetric stretch peak during the oxidation of long alkyd resin under the influence of various mediators. In the drying alkyd resin films 2 mM mediator and 44 U/mL *Mt*L were present.

Vanillic acid doesn't show any significant changes of the 3010 cm^{1} peak, allowing the assumption that no oxidation of the long alkyd resin occurred. For the other mediators the double bond assigned peak decreased clearly over the time of the measurement. A slow decrease was detected for syringaldehyde, acetosyringone and ethylvanillin, indicating a slow oxidation reaction. For vanillin and acetovanillone a fast decrease of the peak was observed. In general an induction time was observed for all mediators. ABTS had the shortest induction time, showing a clear decrease of the peak after 50 hours, while for vanillin and acetovanillone a longer induction time of 72 h was observed.

3.7 Analysis of cross-linked linseed oil and alkyd resin

The LMS catalyzed drying of long alkyd resin and linseed oil was further studied by analyzing the cross-linked sample via ATR-FTIR spectroscopy and gas chromatography. The sample preparation for the analysis was based on the reaction conditions provided during oxygen consumption measurements and the corresponding oxygen consumption measurements can be found in figure 35.

3.7.1 ATR-FTIR spectroscopy

For FTIR analysis, long alkyd resin and linseed oil emulsion were treated with 100 µL substrate, 0.1 mM mediator and 0.03 U/mL laccase in buffer solution and the samples were then lyophilized. The hereby formed cross-linked polymers were taken for analysis. The obtained absorption spectra were normalized and the area of the double bond assigned peak at 3010 cm^{-1} was integrated.

Linseed oil emulsion

After the treatment of linseed oil with ABTS and *Mt*L for 48 hours a solid cross-linked residue was formed. Figure 43 compares the FTIR spectra of the samples treated with laccase and mediator to solely laccase or mediator treated samples. For each spectrum the area of the peak at 3010 cm^{-1} was integrated to compare the results. For the LMS treated linseed oil sample the peak at 3010 cm^{-1} nearly disappears, while for the samples treated with either mediator or laccase the peak is still visible.

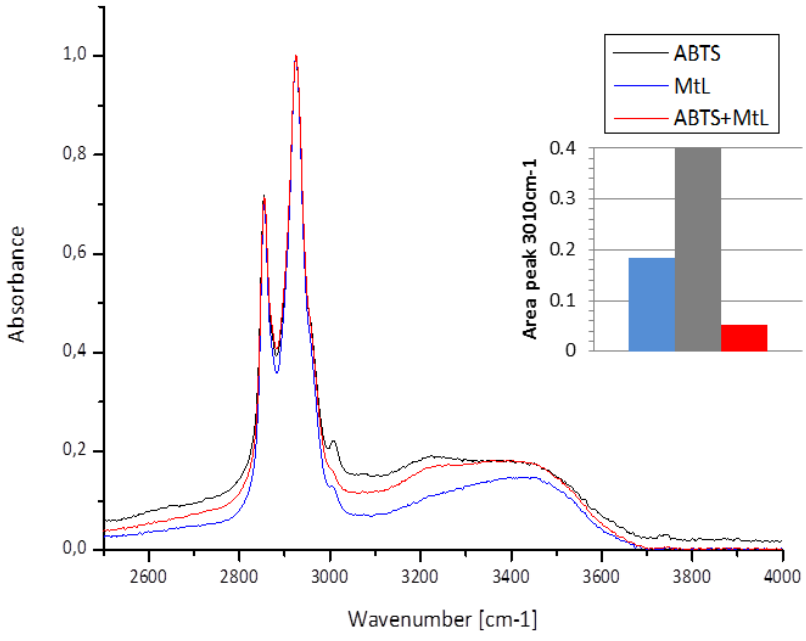


Figure 43: Normalized FTIR spectra obtained for linseed oil emulsion treated with mediator and laccase (red), laccase (blue) and ABTS (black) for 48 h. Only the wavenumber range of interest is shown between 2500 and 4000 cm^{-1}. The area from 2997 to 3022 cm^{-1} was integrated for each spectrum and is compared in the diagram on the right.

Alkyd resin

Long alkyd resin was treated with ABTS and *Mt*L for 24 h or 48 h and the hereby formed solid residue was analyzed by FTIR spectroscopy. The changes of the absorption at 3010 cm^{-1} of the samples treated with a laccase mediator system is compared to solely with *Mt*L or ABTS treated samples. The normalized FTIR spectra are shown in figure 44.

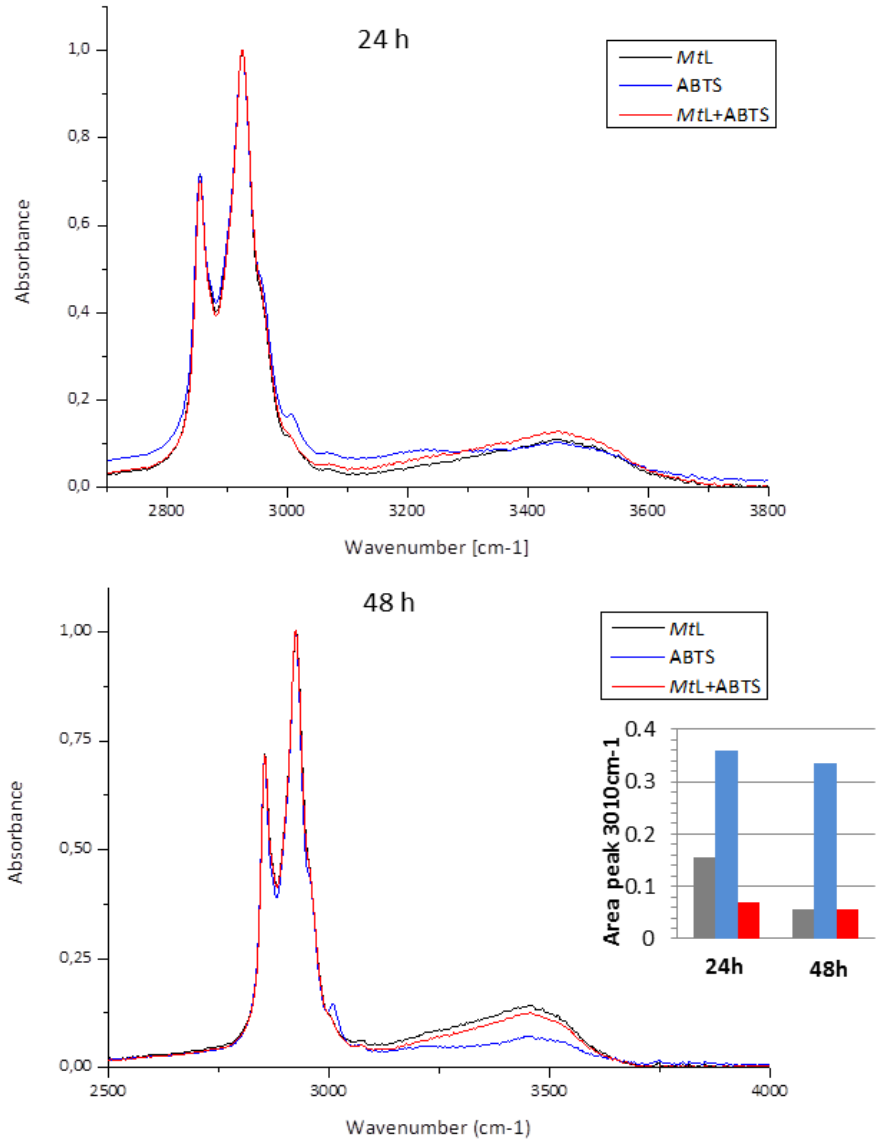


Figure 44: Normalized FTIR spectra obtained from mediator and laccase (red), laccase (blue) and ABTS (black) treated long alkyd resin for 24 h and 48 h. Pictured is only the wavenumber range of interest from 2500 to 4000 cm^{-1}. The area of the peak at 3010 cm^{-1} was integrated in the range of 2996 and 3026 cm^{-1} for each spectrum and is compared in the diagram on the right.

After a 24 hour treatment of long alkyd resin with laccase and mediator (*Mt*L+ABTS) the area of the peak at 3010 cm^{-1} decreases in a higher extent than for the samples treated solely with mediator (ABTS) or laccase (*Mt*L). Although a decrease of the double bond assigned peak was also observed for the samples treated with solely *Mt*L for 24 hours, it can be seen that it is not in the same extent as

for the samples treated with laccase and mediator. However, after treating the samples with *Mt*L for 48 hours the peak at 3010 cm^{-1} decreased to the same level than after treating the samples with laccase and mediator. For solely mediator treated long alkyd resin no significant change of the peak at 3010 cm^{-1} was observed after 24 and 48 hours.

3.7.2 Gas chromatography

The LMS catalyzed drying of long alkyd resin and linseed oil was further studied via gas chromatography. Therefore after treating long alkyd resin and linseed oil for 24 h with a laccase mediator system in an aqueous buffer media, the samples were lyophilized and taken for analysis. It is expected that due to the cross-linking reaction of the unsaturated-fatty acid moieties and the subsequent formation of a polymer, the peak corresponding to triglyceride decreases for both linseed oil emulsion and long alkyd resin.

Linseed oil emulsion

Linseed oil was chosen as a model substrate for alkyd resins because it is the main ingredient of the tested long alkyd resin. The lyophilized cross-linked samples of linseed oil are pictured in figure 45.

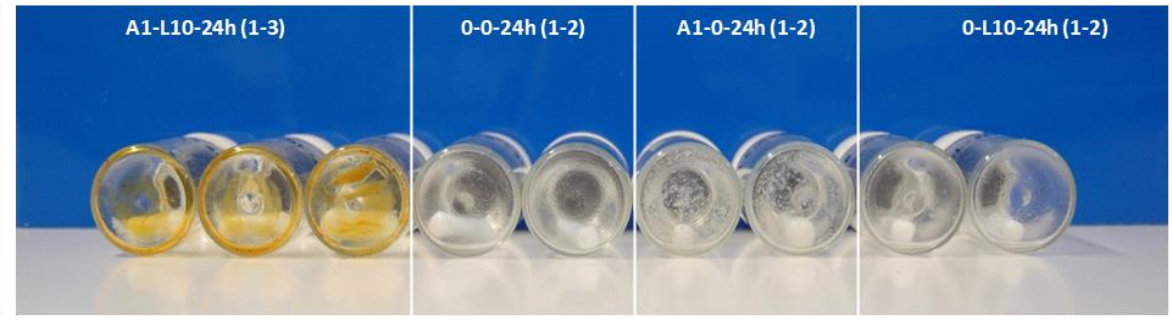


Figure 45: Linseed oil samples after 24 h treatment with laccase (0-L10-24h), mediator (A1-0-24h) or laccase and mediator (A1-L10-24h) and lyophilisation. For the negative sample (0-0-24h) untreated linseed oil emulsion diluted in buffer was mixed for 24 h. For linseed oil samples treated with mediator and laccase a yellow, solid residue was visible.

In figure 46 the gas chromatogram of a linseed oil sample (0-0-0h-1, purple spectrum), which has not been treated with laccase or mediator is shown. The retention time of the internal dodecane standard (blue) was 5.146, of the linoleic acid standard (red) 14.602, and of the trilinolein standard (green) 32.075. All linseed oil samples showed the same pattern, showing four major peaks. The peaks were identified by comparing their retention time with standards. The first peak with a retention time of 4.404 corresponds to the dodecane standard, the second peak is linoleic acid with a retention time of 14.650 and the peaks at a retention time of 31.495 and 32.391 correspond to the trilinolein standard. Because linseed oil is a triglyceride consisting of a mixture of fatty acids (linolenic acid, linoleic acid and oleic acid) two peaks were detected corresponding to the trilinolein standard.

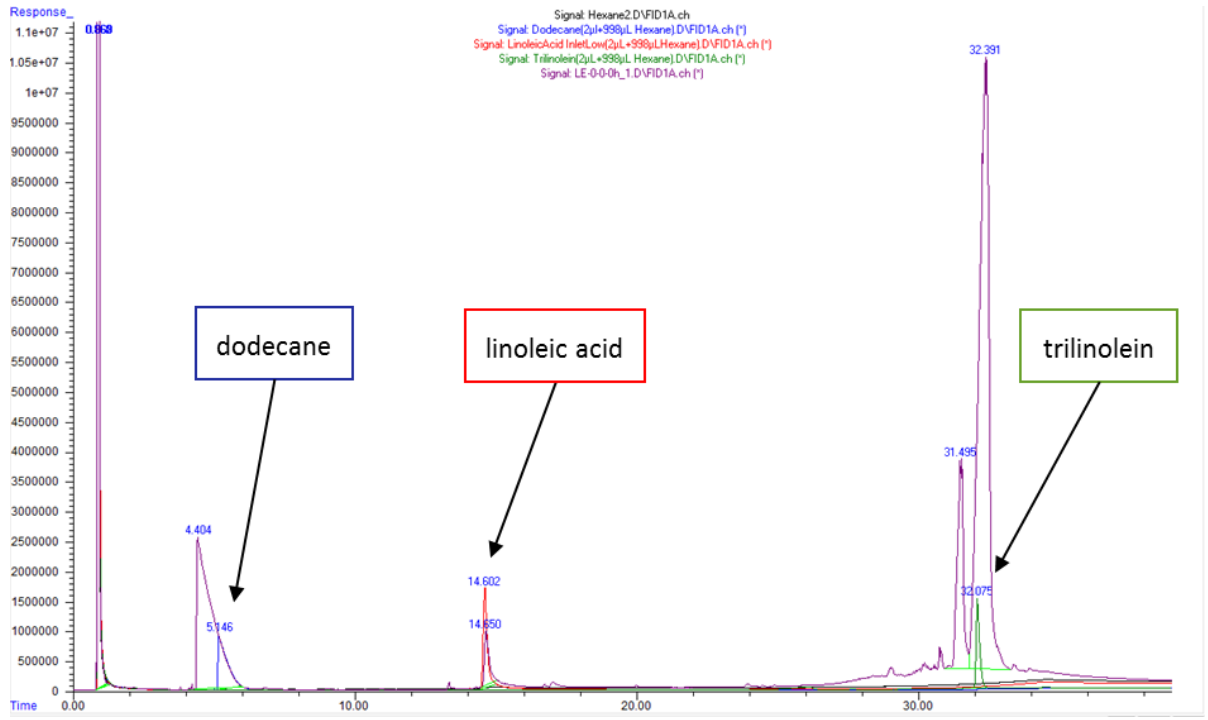


Figure 46: GC chromatograms of untreated linseed oil emulsion (purple) and the standards dodecane (blue), linoleic acid (red) and trilinolein (green).

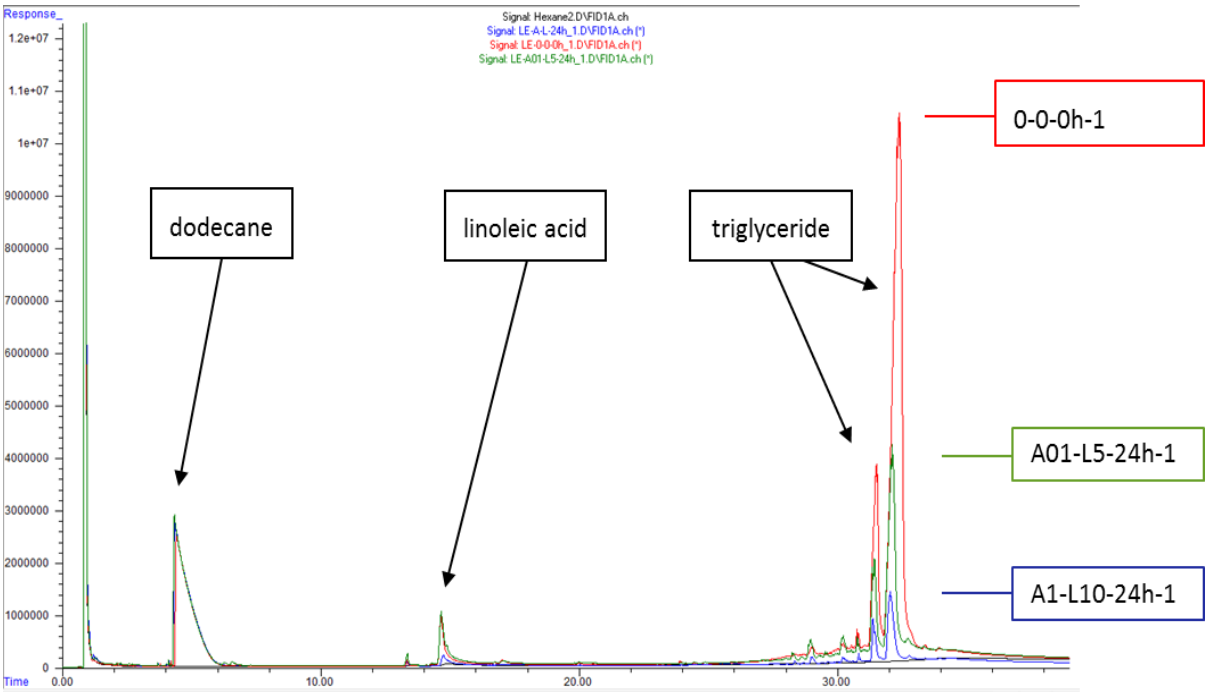
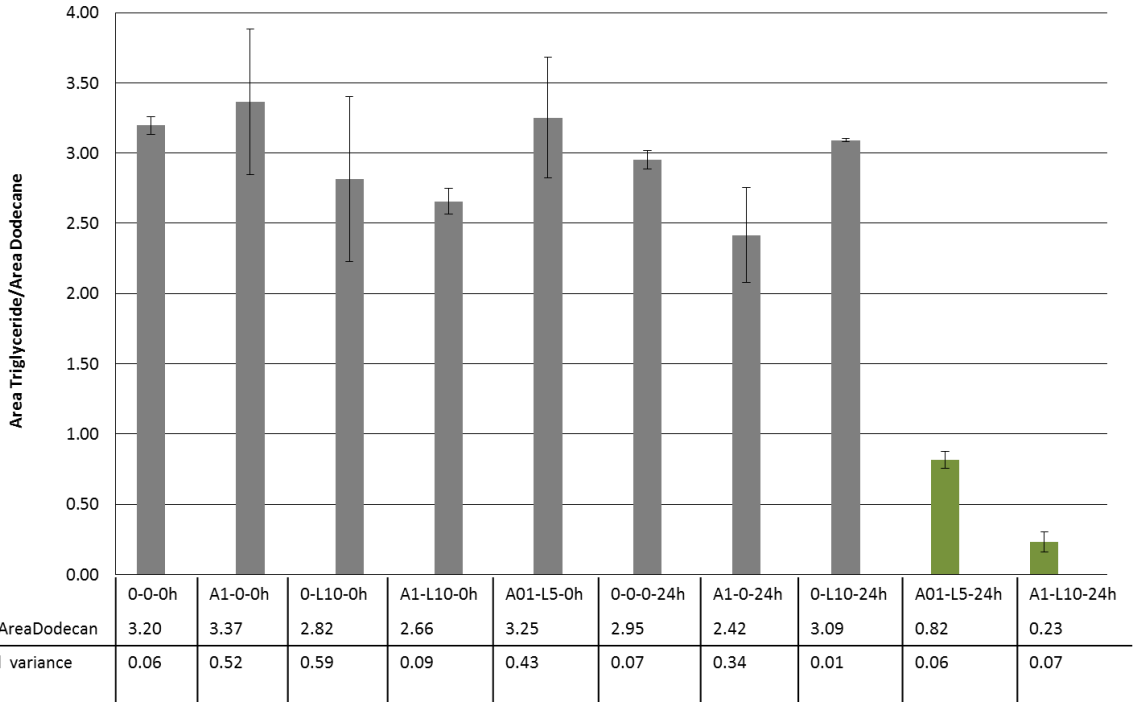


Figure 47: GC chromatograms of untreated linseed oil sample (0-0-0h-1, red) compared to the LMS treated samples A01-L5-24h-1 (green) and A1-L10-24h-1 (blue).

Figure 47 shows the red spectrum of the negative sample (0-0-0h-1), where no enzyme or mediator was added. The spectrum of the linseed oil emulsion treated with 0.1 mM ABTS and 0.03 U/mL laccase is shown in green. The sample of the blue spectrum was treated with a higher concentration of mediator (1 mM) and enzyme (0.05 U/mL). After a 24 h reaction time, a decrease of both triglyceride corresponding peaks was observed (RT 31.495 and 32.391), depending on the used concentration of mediator and laccase.



	0-0-0h	A1-0-0h	0-L10-0h	A1-L10-0h	A01-L5-0h	0-0-0-24h	A1-0-24h	0-L10-24h	A01-L5-24h	A1-L10-24h
AreaTG/AreaDodecan	3.20	3.37	2.82	2.66	3.25	2.95	2.42	3.09	0.82	0.23
standard variance	0.06	0.52	0.59	0.09	0.43	0.07	0.34	0.01	0.06	0.07

Figure 48: Average ratio and standard variance of the in triplicates and duplicates tested linseed oil samples.

For each sample the areas of the two triglyceride peaks were integrated and their sum was calculated. The sum of the triglyceride area was set in ratio with the area of the internal dodecane standard. The average ratio (AreaTG/AreaDodecane) and the standard variance of the triplicates and duplicates were calculated and are shown in

figure **48**. Here you can clearly see that only if mediator and laccase are both present in the mixture, the trilinolein peak decreases. In all other samples no significant decrease of the area ratio could be observed. Further it becomes clear that the decrease of the triglyceride peaks depends on the concentration of mediator and laccase that is used. A higher concentration of mediator and enzyme results in a higher decrease of the triglyceride peaks. The average area ratio of each sample is compared to the area ratio of untreated samples (0-0-0h). For the sample treated with 0.1 mM ABTS and 0.03 U/mL *Mt*L (A01-L5-24h) the area ratio decreases to 26 ± 2 % of the area ratio of untreated sample and for the sample treated with higher concentrations of 1 mM ABTS and 0.05 U/mL *Mt*L (A1-L10-24h) the area ratio decreases to 7 ± 2 % after 24 h incubation.

Table 12: "AreaTG/AreaDodecane" ratio of each sample is compared to the area ratio obtained for untreated linseed oil sample.

Sample	0 h	24 h
0-0	100 ± 2 %	92 ± 2 %
A1-0	105 ± 16 %	75 ± 11 %
0-L10	88 ± 18 %	97 ± 0.4 %
A01-L5	102 ± 13 %	26 ± 2 %
A1-L10	83 ± 3 %	7 ± 2 %

Alkyd resin

Figure 49 shows the long alkyd resin samples used for GC-analysis, after reacting for 24 hours with either laccase or mediator or laccase and mediator. Only for the samples treated with mediator and laccase a solid residue was observed.

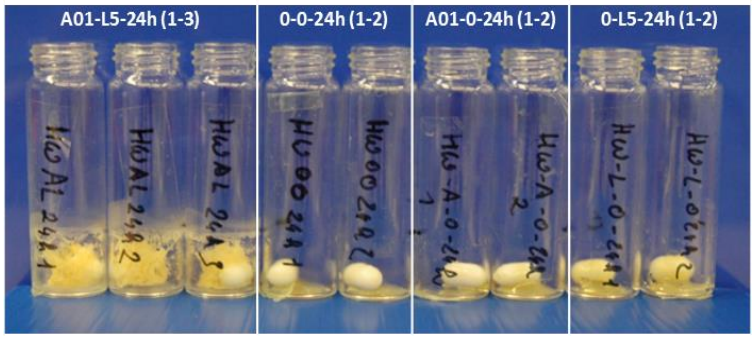


Figure 49: Long alkyd resin samples after a reaction of 24 h with either laccase or mediator or laccase and mediator. For the samples A01-L5-24h (1-3) a solid residue was visible.

Figure 50 shows the gas chromatograms of an untreated long alkyd resin sample (0-0-0h-1, purple), and the standards dodecane (blue), linoleic acid (red) and trilinolein (green). The retention time for dodecane was 5.146, for linoleic acid 14.602 and for trilinolein 32.075. As a typical example of the long alkyd resin samples, the spectrum of the untreated sample 0-0-0h-1 is shown (purple). In comparison to the gas chromatograms of the linseed oil samples, which have only four major peaks, the long alkyd resin samples show several peaks. This was expected, since alkyd resins are very complex systems with a mixture of several components. The peaks of linoleic acid and trilinolein were identified by comparing the retention times with the standards. The peak with a retention time of 4.3 corresponds to the dodecane standard, the peak with the retention time of 14.7 is linoleic acid, and the peaks with the retention times 31.6 and 32.2 correspond to the trilinolein standard.

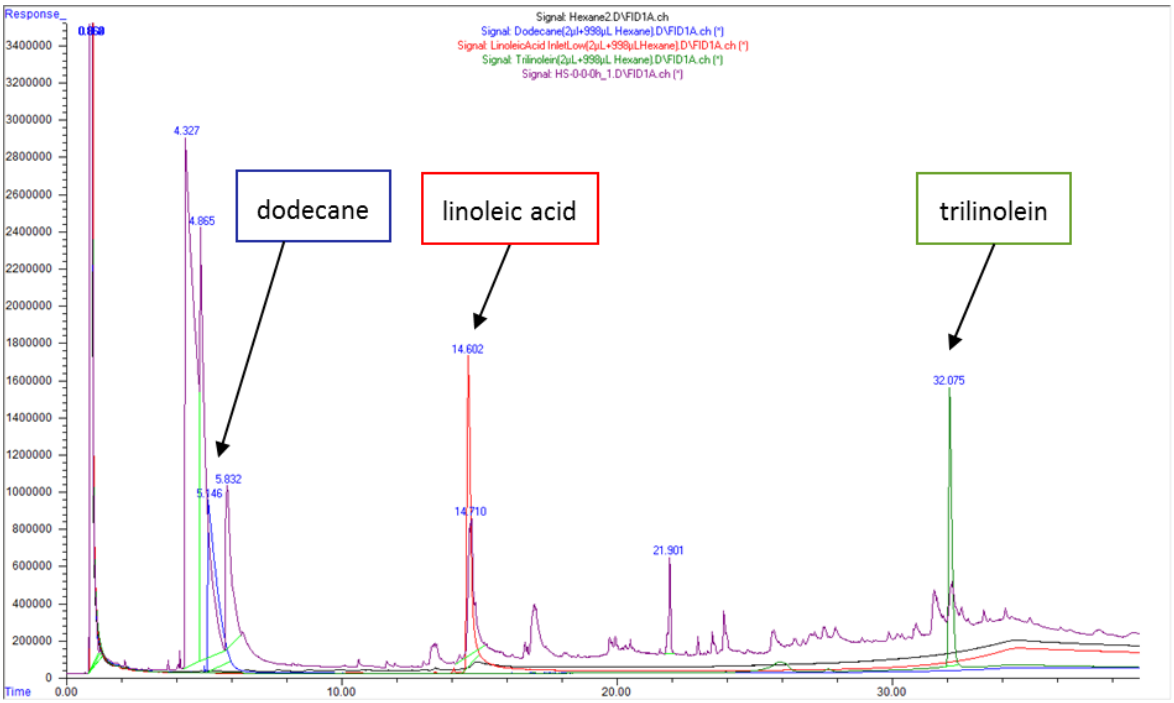


Figure 50: Gas chromatograms of untreated long alkyd resin sample (0-0-0h-1, purple), the dodecane standard (blue), linoleic acid standard (red) and trilinolein standard (green).

Figure 51 shows in green the chromatogram of long alkyd resin sample treated with mediator and laccase for 24 hours (A01-L5-24h-1). The red and blue spectra are untreated long alkyd resin samples, analyzed after a reaction time of 0 h (blue) and 24 h (red) respectively.

The areas of the two triglyceride peaks were integrated and the sum was set in ratio with the area of the internal dodecane standard. The average ratio and the standard variance of the triplicates and duplicates are given in figure 52. In none of the samples treated with laccase, mediator, or laccase and mediator, a clear decrease of the trilinolein or the linoleic acid corresponding peaks were observed.

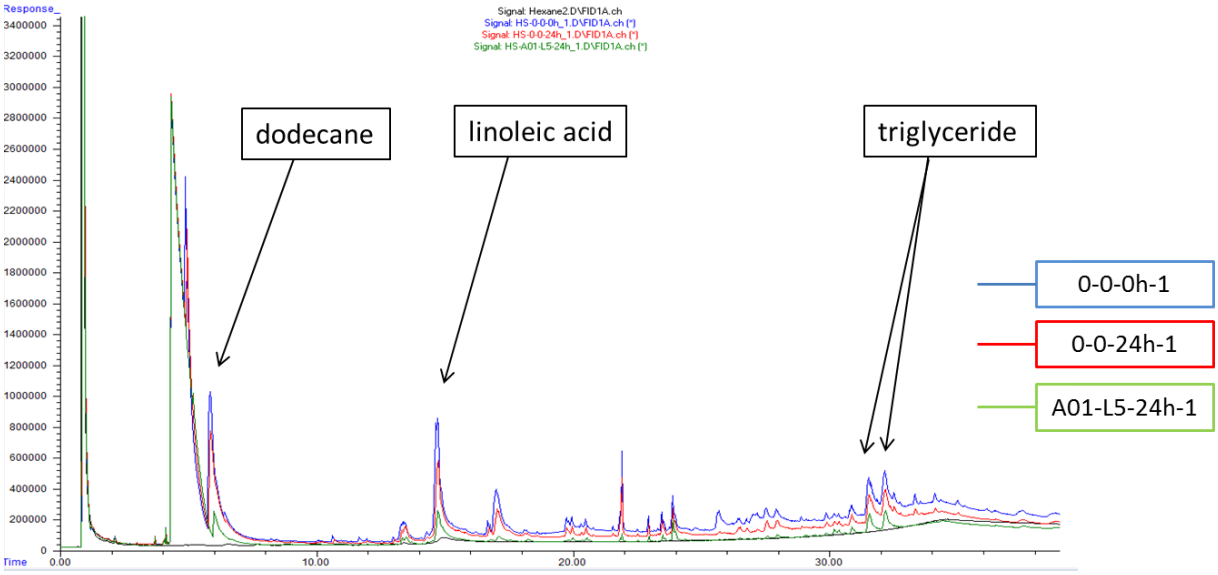


Figure 51: GC chromatograms of untreated long alkyd resin sample (0-0-0h-1, blue) and (0-0-24h, red) compared to the LMS treated sample (A01-L5-24h-1, green). The two main triglyceride peaks of long alkyd resin had a retention time of 31.564 and 32.156.

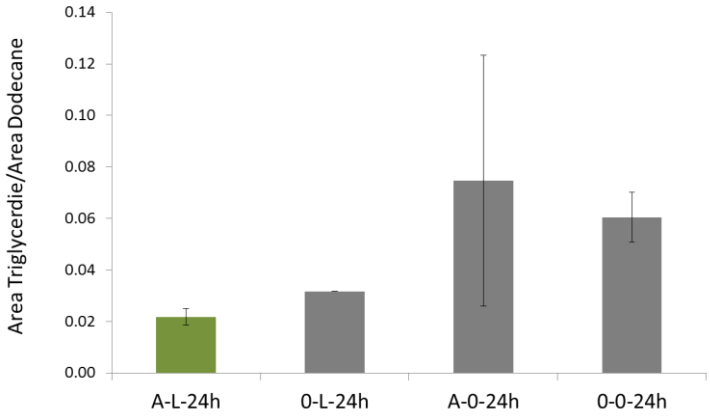


Figure 52: Average of the two main triglyceride peaks of long alkyd resin set in ratio with the area of the dodecane standard. The samples have been treated with mediator and laccase (A-L-24h), laccase (0-L-24h) or mediator (A-0-24h) and the negative sample (0-0-24h) lacked both laccase and mediator. The reaction time for all samples was 24 hours. Further, the standard variance of the triplicates is given.

4 Discussion

This thesis is part of a project with the objective to establish an enzymatic method to dry alkyd resins in order to substitute heavy metal based siccatives in water-borne alkyd resin paints. In previous project studies, it could be shown that a laccase mediator system (LMS) efficiently catalyzes the cross-linking of unsaturated fatty acid side chains in alkyd resins using *Trametes hirsuta* laccase and ABTS or HBT as a mediator [1]. The methods used to study and evaluate the LMS catalyzed cross-linking reaction were oxygen measurements, FTIR analysis and drying recorder tests. Oxygen consumption confirmed cross-linking of alkyd resin not only in aqueous media but also in drying alkyd resin films. On a molecular level the cross-linking reaction was studied by FTIR analysis, where a decrease of the double bond associated peak, caused by the oxidation of the unsaturated fatty acid side chains, was observed. Drying recorder tests, the gold standard in the coating industry, were used as an additional tool to evaluate the efficiency of the drying reaction. Further, an increase of the molecular weight of the long alkyd resin was quantified using gel permeation chromatography. To sum up, a laccase-mediator system was introduced as an effective catalyst for the cross-linking of long alkyd resins, with the potential to replace cobalt based siccatives in alkyd resin paints. [1]

The objective of this thesis is to continue previous project studies and find ways to improve the laccase mediator catalyzed cross-linking of alkyd resins. In order to be able to improve the drying reaction, it is necessary to further investigate the drying process of alkyd resins and determine influencing factors. Therefore, the previous established methods (e.g. oxygen measurements, FTIR analysis and drying recorder tests) to follow and evaluate the drying process were used and adapted if needed. Gas chromatography analysis is introduced in this thesis as an additional method to study the cross-linking product after treatment of alkyd resin with a laccase mediator system. In regards to a final application of a LMS in alkyd resin paints at an industrial scale, this study also addresses application specific points. One important point was to investigate the storage stability of the mediator and laccase mixed into the alkyd resin coating. The next important point was to find alternative mediators to ABTS and HBT. The synthetic mediators ABTS and HBT were used in previous studies as laccase mediators for the drying of alkyd resins. However, because of high costs and possible toxicity of these synthetic mediators the application at an industrial scale might be hindered [68].

In a first step the pH optimum, protein concentration and activity of the enzymes were determined. The laccases used in this study to catalyze the cross-linking of alkyd resins are fungal laccases *Trametes hirsuta* and *Myceliophthora thermophila.* The activity of *Th*L and *Mt*L was

determined measuring the oxidation rate of ABTS. The highest activity for *Th*L was measured at pH 4 and the laccase activity steadily decreases with higher pH. These results are in agreement with pH profiles of *Th*L found in the literature using the non-phenolic substrate ABTS [25]. The pH optimum of the commercial available *Mt*L (40035, 51003) from Novozymes was determined at pH 7. For both laccase, *Th*L and *Mt*L, a broad pH-spectrum was determined, which is an advantage because during the hardening of the internal emulsified alkyd resin a pH shift occurs. The tested long alkyd resin is adjusted to a pH 7 by adding dimethylethylamine and during the hardening of the alkyd resin film the amine evaporates, which changes the pH in the film to more acid values.

The performed ABTS assay is a simple, rapid and well known method to determine the pH optimum of laccases. However, this method does not involve the alkyd resin as a substrate and therefore might not adequately mimic the processes in the drying of alkyd-resins [65]. Because of that, the pH dependency of the laccase-mediator catalyzed cross-linking reaction was further studied by measurements of the oxygen uptake in aqueous media. In order to identify the pH at which *Th*L and *Mt*L perform best in the drying process, the amount of oxygen consumed during the cross-linking reaction of long alkyd resin was measured comparing various pH conditions. Measurements of the oxygen consumption were already used in previous project studies to observe the LMS catalyzed drying reaction of alkyd resins in aqueous media as well as alkyd resin films. By comparing the amount of oxygen consumed during the reaction it is possible to evaluate the efficiency of the cross-linking reaction. Next to pH dependency studies oxygen consumption measurements were used in this work to identify laccase mediators and to test different laccase substrates.

For both enzymes, the performed oxygen consumption measurements revealed that the laccase-mediator catalyzed cross-linking of long alkyd resin is influenced by the pH conditions. For *Th*L the highest amount of oxygen consumed was determined at pH 6, the least at pH 4. For *Mt*L the highest amount of consumed oxygen was measured at pH 7. Judging by the amount of consumed oxygen, the LMS catalyzed cross-linking reaction works best at pH 6 using *Th*L and pH 7 using *Mt*L.

There can be various reasons for the observed pH dependency of the LMS catalyzed cross-linking reaction. Principally any pH induced mechanistic or structural changes in either the alkyd resin emulsion, O_2, laccase (*Th*L, *Mt*L) or mediator (ABTS) could contribute to the observed pH dependency. It has to be considered that the laccase was diluted to fit an activity of 0.03 U/mL in the total reaction volume at the given pH, based on the prior performed activity measurements using an ABTS assay. In this way the contribution of pH induced changes of the laccase and mediator is reduced, this lays the focus on the alkyd resin emulsion. The alkyd resin emulsion might be influenced by the pH, in a similar way as it was observed for lipid oil-in water emulsions [72][73]. At basic pH the droplet charge of lipid oil-in-water emulsions is more negative and the oxidation

potential of the lipid emulsions increases [73]. In drying studies of alkyd resin emulsions using metal ions as catalysts, a pH dependent change of the droplet charge was measured as well [17][74]. At basic pH the metal ions were attracted to the negative charge of the interface of alkyd resins and a faster drying of the alkyd resin was observed [17]. The pH induced changes of the alkyd resin might as well have an influence on the cross-linking reaction catalyzed by the laccase mediator system. It could be, that the oxidized mediator or the laccase is more attracted to a negative charged alkyd resin surface, which explains the highest oxygen consumption rate for *Mt*L at pH 7 and *Th*L at pH 6. In previous studies, the isoelectric point of *Trametes hirsuta* laccase was determined to be pH 7 [23]. At pH conditions below its isoelectric point, the laccase carries a net of positive charge. When assuming that the alkyd resin emulsion droplets are more negative charged at higher pH, the positive charged laccase might stick to the surface of negative charged alkyd resin droplets with increasing pH, which results in faster oxidation of the alkyd resin.

Of course these are just possible explanations and further investigations, such as measurements of the droplet charge and size of the alkyd resin emulsion in dependence of the pH or electrochemical studies of the mediator by means of cyclic voltammetry need to be done. Based on the performed oxygen consumption measurements, we can conclude that the optimal pH for the cross-linking of alkyd resin catalyzed with a laccase mediator system is pH 6 for *Th*L and pH 7 for *Mt*L, using ABTS as a mediator, and long alkyd resin as a substrate. However, it has to be considered that any changes of the mediator, laccase or substrate make it necessary to rerun the oxygen consumption measurements to determine the optimum pH for the cross-linking reaction under the given circumstances.

After characterisation of the two laccase types and studies of the pH dependency of the cross-linking reaction, it was decided to focus on using *Myceliophthora thermophila* laccase for further tests. The primary reason for this decision was that most water-based alkyd resins have a pH between 7.2 and 8.5 [75], fitting the pH optimum of M*yceliophthora thermophila* laccase (40035, 51003). Additionally, the tested M*yceliophthora thermophila* laccase has a higher activity than *Trametes hirsuta* laccase at their respective pH optimum.

To determine the oxidation potential of M*yceliophthora thermophila* laccase in combination with ABTS towards different substrates, oxygen consumption measurements were performed. Studies of oil-in water lipid emulsions have shown that the chemical structure of lipids, more specific the grade of unsaturation of the fatty acid moieties, influences their oxidation potential [72]. The higher the grade of unsaturation of the fatty acid side chains, the higher was the oxidation potential

of the lipid. Additionally, the oxidation rate of unsaturated fatty acids is influenced by the position of the double bond [72], in the way that the susceptibility towards oxidation decreases the closer the double bond on the fatty acid is to its methyl end [72]. To test the influence of the structure of the fatty acid side chains in alkyd resins, several oil-in water emulsions (e.g. linseed oil, linoleic acid and trilinolein) were tested for their ability to be oxidized by a laccase mediator system.

As a negative control the saturated triglyceride tributyrin was tested as substrate. As expected no oxygen was consumed, therefore no cross-linking reaction took place because of the lack of double bonds. Also, no oxygen consumption was observed using inactive laccase. The highest oxygen consumption rate was measured using linseed oil, a triglyceride with linolenic fatty acid (C18:3) as main constituent. For trilinolein, a triglyceride with linoleic acid as sole constituent (C18:2), significantly less oxygen was consumed than for linseed oil. These results are in agreement with published lipid oxidation studies, where lipid emulsions with higher contents of polyunsaturated fatty acids are more likely oxidized [72]. The tested linseed oil emulsion has a higher potential to be oxidized than trilinolein, because of the additional fatty acid double bonds of the linolenic acid side chains.

Further, the used linseed oil emulsion contains 14-26 % free linoleic acid in contrast to pure trilinolein, which might also enhance the oxidation ability of the laccase mediator system towards the substrate. In previous drying studies of emulsified oils with metal catalysts, it was claimed that free fatty acids act as pro-oxidants [73]. It was observed that an addition of free fatty acids to oil emulsions increases the formation of hydroperoxides and hexanal, which are primary and secondary lipid oxidation products [73]. Two reasons were given for this observation: A significant decrease of the droplet surface charge of the emulsions by adding the free fatty acids, such as linoleic, linolenic or oleic acid increases the attraction of metal catalysts. Further, a co-oxidizing ability of free fatty acids was suggested. The pro-oxidant effect of free fatty acids is further supported in this study by significant higher oxygen consumption measured for linoleic acid emulsion compared to trilinolein emulsion and methyl linoleate emulsion, which both lack a free carboxylic group (-COOH).

Also, in previous oxidation studies of fatty acids it was claimed that conjugated fatty acids undergo a different oxidation mechanism than non-conjugated fatty acids [76]. It was suggested that the oxidation of non-conjugated fatty acids is initiated by the abstraction of bis-allylic hydrogen atoms, which have relatively low C-H dissociation energy, resulting in a radical chain reaction and cross-linking [76]. For conjugated unsaturated fatty acids a different oxidation mechanism where less oxygen is consumed, is suggested [76]. This could also be observed in this study, where less oxygen for the castor oil based alkyd resin was consumed than for the long alkyd resin. The only difference in their composition is the conjugation of their main fatty acid constituent linoleic acid; while castor oil

based alkyd resin has a conjugated linoleic acid as its main constituent, long alkyd resin consists of non-conjugated linoleic acid.

So far it was shown that a laccase mediator system efficiently catalyzes the drying of alkyd resin using ABTS or HBT as mediator [1]. Admittedly, ABTS and HBT are synthetic mediators with potential health risk and relatively high costs [68]. Therefore, it is important to find alternatives to these synthetic mediators, which are suitable for industrial application in alkyd resin coatings.

In general, the search for natural compounds as laccase enhancers is a huge biotechnological topic, as laccases are already used in industrial applications with the potential to be applied in many more processes. A lot of publications already focus on the search for natural compounds as new laccase enhancers, and some natural compounds have been successfully identified as laccase mediators [27][30][38]. These laccase enhancers are mainly phenolic mediators involved in the degradation of lignin. The compounds tested in this study are vanillin, vanillic acid, ethyl vanillin, acetovanillone, syringaldehyde and acetosyringone and have been already mentioned in the literature as laccase enhancers [27][38]. Their chemical structure is based on lignin polymers, and all of them had a phenolic ring, with either alkyd, ketone or an acid side chain.

The methods used to screen for suitable laccase mediators are oxygen consumption measurements, drying recorder tests and FTIR measurements. At the beginning of the mediator screening, oxygen consumption measurements were performed, because those measurements can be performed quickly, with low amounts of mediator, laccase and alkyd resin. Another important advantage of this method is the high sensitivity and reproducibility. This first screening method performed in aqueous media was used to identify efficient laccase enhancers judging by the consumption of oxygen, followed by mediator screenings in drying alkyd resin films using DTR and FTIR measurements. Drying time recorder measurement is a widely used and accepted method in coating industries to evaluate the curing stages in alkyd resin films. However, this method has its limits because it shows low sensitivity and small differences of the drying ability are not detectable. FTIR-analysis allows the characterization of the drying process on a molecular level by observing the decrease of the double bond assigned peak at 3010 cm^{-1}.

The natural compounds vanillin, syringaldehyde, ethyl vanillin, acetosyringone and acetovanillone where identified by oxygen consumption measurements as laccase enhancers. For all of these compounds clear oxygen consumption in the range of 80 to 98 % was determined over time, indicating efficient cross-linking of the long alkyd resin. Most promising oxygen consumption rates were determined for acetovanillone, which were similar to the oxygen consumption profile

determined using ABTS as mediator. Vanillic acid was identified as ineffective laccase mediator due to the lack of oxygen consumption over time. The oxygen consumption profile of vanillic acid and the negative control with sole *Mt*L treatment are very similar, indicating the lack of cross-linking of the long alkyd resin.

All compounds identified as laccase enhancers by the oxygen consumption measurements, were also proven to catalyze the drying in long alkyd resin film determined by DTR tests and FTIR analysis. Vanillic acid is an example of an ineffective mediator, in its case a lack of oxygen consumption, a bad drying performance by DTR and no decrease of the double bond assigned peak at 3010 cm^{-1} peak over time was observed. Considering the results of all three screening methods, acetovanillone and vanillin were identified laccase enhancers with similar drying results to the synthetic mediator ABTS.

All in all, the results of the performed oxygen consumption measurements correspond to the results of the DTR and FTIR measurements. Nevertheless, a sufficient evaluation of the drying potential of the laccase mediator system in alkyd resin films has to consider the results of all three methods. It is important to keep in mind that the conditions of the cross-linking reaction in solution differ from the drying film. One important point is that the diffusion of oxygen through the drying surface of the alkyd resin film is hindered with advancing curing stage. In the case of the oxygen consumption measurements, the influence of oxygen diffusion is kept at the same level by constant stirring of the aqueous reaction media during the reaction. Another important point to be considered is the distribution of the laccase mediator system in the drying alkyd resin film. In the course of the drying reaction the distribution of the LMS might change, while in the aqueous reaction media there is an even distribution of the LMS throughout the whole reaction process. Furthermore, in the course of the drying of the alkyd resin film, the pH of the film shifts due to the evaporation of dimethylamine, affecting the activity of the laccase. Whereas, in the cross-linking reaction carried out in buffered mediator solutions the pH is stable during the whole reaction process.

The laccase mediator catalyzed cross-linking reaction of alkyd resin was further investigated by analysis of the cross-linked linseed oil and alkyd resin using FTIR analysis and gas chromatography. Because of the complexity of the long alkyd resin, linseed oil emulsion was chosen as a model substrate.

Via FTIR analysis a clear decrease of the double bond assigned peak at 3010 cm^{-1} was observed when treating linseed oil with a laccase mediator system for 48 h. For the linseed oil samples treated exclusively with *Mt*L the double bond assigned peak also decreased but not in the same extend. In contrast, the FTIR analysis results of the more complex cross-linked long alkyd resin samples were

not so clear. After a 24 h treatment of long alkyd resin with a laccase mediator system the double bond assigned peak decreased in a higher extend than for alkyd resin treated exclusively with laccase or mediator. However, after treating the sample for 48 hours exclusively with laccase the peak area at 3010 cm^{-1} decreased nearly in the same extend than for samples treated with laccase and mediator.

Gas chromatography analysis of the cross-linked linseed oil product confirmed a decrease of the triglyceride peak due to the LMS catalyzed cross-linking reaction of the unsaturated-fatty acid side chains in linseed oil. Also, the degree of the area decrease of the triglyceride peak depends on the concentration of laccase and mediator used to catalyze the cross-linking reaction of linseed oil. By increasing the laccase and mediator concentration a higher decrease of the triglyceride peak area was observed after a reaction time of 24 hours. However, gas chromatographic analysis of the more complex long alkyd resin samples did not show a clear decrease of the trilinolein corresponding peaks. This is most likely caused by the complexity of the alkyd resin samples, which makes the analysis of the cross-linked product more difficult than for the model substrate linseed oil emulsion. For further studies of the laccase mediator catalyzed cross-linking reaction gas chromatography analysis is only applicable using linseed oil as model compound.

It has been observed that storing laccase and mediator in the long alkyd resin has an adverse effect on the drying performance. For the enforcement of the practical application of a laccase mediator system as catalyst in alkyd resin paints, the stability of the enzyme and mediator during storage is of high importance and has been investigated in this study.

In a first step, the chelating agent DMEA, which is present in the long alkyd resin in a concentration of 0.08 % w/w, was tested for potential inhibitory effects on the *Mt*L activity. DMEA is a copper chelator, which can cause a loss of catalytic activity of the laccase due to selective removal of type one copper [77]. In previous preformed laccase activity inhibition tests the chemicals acticide, tin(II)ethylhexanoate and triphenyl phosphite, were identified as inhibitants on the *Th*L activity. As a consequence these compounds have been eliminated from the formulated alkyd resin and a model alkyd resin (called long alkyd resin, HSW 323/23c) was produced for future studies [1]. To determine potential inhibition of DMEA on the activity of *Myceliophthora thermophila* laccase, activity measurements were performed in the presence of 0.08 % of the chelating agent DMEA. The laccase activity inhibition test did not indicate an inhibitory effect of a 0.08 % DMEA concentration, which is the same concentration present in the model alkyd resin. However, alkyd resin paints are complex

mixtures of various compounds, which might have an inhibitory effect on the laccase activity and further tests need to be performed testing these substances.

Generally, a loss of drying performance is a known phenomenon in alkyd resin paints after a certain storage period. It is known that the drying time of stored alkyd resin paints compared to freshly prepared alkyd resins gradually increases depending on the storage time [17]. Weissenborn & Motiejauskaite published a systematic study of the drying properties of alkyd resins and found out that the grade of the drying loss upon long-term storage is influenced by properties of the alkyd resin such as the pH of the emulsion, the way of resin neutralisation, the presence of pigments as well as the type of drier. For all tested alkyd resin emulsions a loss of drying due to long term storage was observed [17]. Further, during the storage of alkyd resin emulsions the pH decreased steadily from neutral down to about 4-5 caused by acid hydrolysis of ester linkages and formation of weak carboxylic acids [17]. Therefore, besides the possibility of laccase inhibitors present in the formulated alkyd resin, a steady decrease of the pH during storage of the alkyd resin might cause an increase of drying time. In this case, the laccase activity decreases due to pH changes of the alkyd resin during storage resulting in a degradation of drying properties. To investigate this issue the loss of drying performance of the laccase mediator system was investigated more closely using oxygen consumption measurements. Hereby, changes of the laccase activity and mediator properties over the time of storage were investigated. Laccase and mediator were mixed into the long alkyd resin and stored over a period of time. The activity of *Myceliophthora thermophila* laccase decreases clearly after a storage time of 25 days in the long alkyd resin, visual in a more than 20 % lower oxygen consumption. After 10 and 17 days no significant decrease of oxygen consumption could be observed. The results of the oxygen consumption measurements showed that the mediator is not influenced by the storage of 25 days in the long alkyd resin, as the oxygen consumption rate did not decrease. Therefore, the decrease of the laccase activity during storage in the long alkyd resin is most likely the cause for an increase of the drying time as it was also observed by drying time recorder measurements.

In order to stabilize the laccase activity during storage it is important to find ways to stabilize the pH of the alkyd resin and to identify possible laccase inhibitors present in the alkyd resin paint. However, this is a huge challenge, due to the complexity of alkyd resin paints. Therefore, it might be easier to develop a product where the laccase is stored separately and is mixed into the alkyd resin paint before application.

5 Conclusion

The objective of the project, this thesis is embedded in, is to develop a method to enzymatically cross-link the unsaturated fatty acid moieties in alkyd resins.

In this thesis, gas chromatography analysis was introduced as a new method to study the cross-linked linseed oil after treatment with a laccase mediator system. Via gas chromatography analysis a decrease of the triglyceride corresponding peaks confirmed the cross-linking of linseed oil. All in all, it has been clearly proven that a laccase in combination with different natural mediators catalyzes the drying of alkyd resins and has the potential to substitute heavy metal based siccatives in water-borne alkyd resin paints. However, in regards to future industrial application the drying process needs to be optimized to make the laccase mediator system more competitive to the cobalt based siccatives. In order to optimize the drying process, ideal conditions for the laccase mediator system catalyzed drying reaction need to be provided.

According to the performed oxygen consumption measurements, the ideal pH for the LMS catalyzed cross-linking reaction of the tested long alkyd resin is pH 7. Therefore *Mt*L was chosen for further drying tests because of its optimum pH of 7. However, in the drying alkyd resin film a pH shift occurs causing a decrease of the laccase activity. A decrease of the laccase activity was also observed after storage of laccase and mediator in the long alkyd resin for 25 days. Genetic modification has a high potential to optimize the laccase in terms of increasing the laccase activity in the alkyd resin or make it more resistant against compounds present in the alkyd resin.

Another factor that influences the oxidation reaction of unsaturated fatty acid side chains in alkyd resin emulsions is the supply of oxygen. During the drying of alkyd resin films, the diffusion of oxygen decreases and oxygen supply becomes a limiting factor of the cross-linking reaction [1]. Future research should include detailed studies of the diffusion of oxygen and the distribution of the laccase mediator system during the drying of alkyd resin film.

One important point to optimize the reaction was to find alternative laccase mediators to the synthetic mediators ABTS and HBT. In this study acetovanillone and vanillin have been identified by a mediator screening using oxygen consumption measurements, FTIR-analysis and drying recorder tests, as most promising alternative laccase mediator to the synthetic mediators ABTS and HBT. In future works further drying studies using the hereby identified laccase mediators should be performed.

Above all, a good understanding of the reaction process and involved mechanisms makes it possible to further optimize of the LMS catalyzed cross-linking reaction. However, it has to be considered that

a description in every detail of the complex drying reaction of alkyd resins and its influencing factors is not impossible [3]. For a further description of the reaction process, a combination of several analytical methods is necessary. Not yet applied methods to observe the LMS catalyzed drying reaction involve concentration measurements of formed intermediates, such as hydroperoxides or the formation of end products, such as alcohols or ketones which are indicators for the oxidation process [72].

6 Declaration

STATUTORY DECLARATION

I declare that I have authored this thesis independently, that I have not used other than the declared sources / resources, and that I have explicitly marked all material which has been quoted either literally or by content from the used sources.

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Graz, am …… ……..

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8 Abbreviations

%	percent
°C	degree Celsius
µg	microgram
µl, µL	microlitre
µm	micrometer
ABTS	2,2‘-azinobis-(3-ethylbenzothiazoline)-6-sulphonate
BCA	bicinchoninic acid
BSA	bovine serum albumin
cm	centimeter
d	optical path leng
ddH_2O	ultrapure water
dH_2O	distilled water
e.g.	for example
f	dilution factor
FTIR	fourier transform infrared spectroscopy
GC	gas chromatography
GPC	gel permeation chromatography
h	hour
H_2O	water
HBT	1-hydroxybenzotriazole
HCl	hydrochloric acid
HPI	N-hydroxyphthalimide
kDa	kilodalton
K_m	Michaelis constant
L	litre
LMS	laccase mediator system
M	mol per litre
mg	miligramm
mg/mL	milligram per millilitre
min	minute
mL	milliliter

mM	millimole per liter
*Mt*L	*Myceliophthora thermophila* laccase
MW	molecular weight
NHA	N-hydroxyacetanilide
rpm	rotations per minute
RT	room temperature
SDS	sodium dodecyl sulfate
*Th*L	*Trametes hirsuta* laccase
TRIS	2-Amino-2-hydroxymethyl-propane-1,3-diol
*Tv*L	*Trametes villosa* laccase
U	Units
U/mg	units per miligramm
U/ml	units per mililitre
V	Volt
V_0	volumetric enzyme activity
VLA	violuric acid
V_{max}	maximum reaction rate
V_{sample}	sample volume
V_{tot}	total volume
Δabs	change of absorbance
Δabs/min	change of absorbance per minute
ε	molar extinction coefficient
ε_{ABTS}	molar extinction coefficient of ABTS
NaF	natriumfluoride
VOCs	volatile organic compounds
NH_4Ac	ammonium acetate

9 Equipment

analytic balance	Sartorius 2004 MP
analytic balance	KERN PB
balance	DENVER INSTRUMENT S-4002
centrifuge	Eppendorf mini spin
centrifuge	Hettich EBA 3S
electrophoresis power supply	BIO-RAD POWER PAC 1000
fiber-optic oxygen meter	MICROX TX3, PreSens Precision Sensing Gmb
film applicator	Erichsen, Quadruple Film Applicator Model 360
FT-IR Spectrometer	Perkin Elmer, Spectrum 100
GC-column	Zebron Inferno ZB-5HT, Phenomenex
GC-FID	Agilent Technologies, 7890A GC-FID System/5975C VL MSD Triple-Axis Detector
magnetic stirrer	VARIOMAG Electonicrührer MULTIPOINT HP
oxygenmeter	RANK BROTHERS LTD. DUAL DIGITAL MODEL 20
pH-meter	METTLER TOLEDO Seven Easy
photometer	HITACHI U-2001 Spectrophotometer
pipette	Carl Roth GmbH
pipette	Soccorex, ACURA 825
plate reader	TECAN infinite M200
scanner	HP Scanjet 4890
shaker	INFORS HT Multitron
thermomixer	Eppendorf Thermomixer comfort
ultrasonic bath	Elma TRANSSONIC DIGITAL S
ultrasonic bath	BANDELIN SONOREX SUPER RK 102H
vortex	IKA VORTEX GENIUS 3
waterbath	B. BRAUN THERMOMIX 1420
well plates	Greiner bio-one

10 Chemicals

Acetic acid	Carl Roth GmbH
Acetosyringone	Sigma-Aldrich
Acetovanillone	Sigma-Aldrich
Acrylamide bis-acrylamide 40 % solution	Sigma Aldrich
Albumin, bovine serum	Sigma
BC Assay Protein Quantification Kit	BioRad
2,2‘-azinobis-(3-ethylbenzothiazoline)-6-sulphonate	Sigma
Bradford Reagent	Carl Roth GmbH
CaF2 Crystal window	Sigma
Castor oil based alkyd resin (PRE6/15)	Allnex, Austria
Cobalt oil drying agent	Allnex, Austria
Coomassie Brilliant Blue R-250	BIO-RAD
Ethylvanillin	Sigma
1-Hydroxybenzotriazole hydrate	Aldrich
Linoleic acid	Sigma-Aldrich
Linseed oil	Allnex, Austria
Long alkyd resin (HSW 323/23c, inhibitor free)	Allnex, Austria
Methyl linoleate	Sigma-Aldrich
Myceliophthora thermophila Laccase	Novozymes
NaF	Sigma-Aldrich
o-Vanillin	Sigma-Aldrich
SDS-PAGE Molecular Weight Standards, Broad Range	Bio Rad
Sodium acetate	Carl Roth GmbH
Sodium hydrogen phosphat	Carl Roth GmbH
Syringaldehyde	Sigma-Aldrich
TEMED	Carl Roth GmbH
Trametes hirsuta Laccase	Institute of Environmental Biotechnology of the Graz University of Technology
Tributyrin	Nu-chekprep, Inc.
Trilinolein	Nu-chekprep, Inc.
Tris	Carl Roth GmbH
Tween 20	Sigma
Vanillic acid	Sigma-Aldrich

11 Appendix

Specific activity of *Th*L and *Mt*L

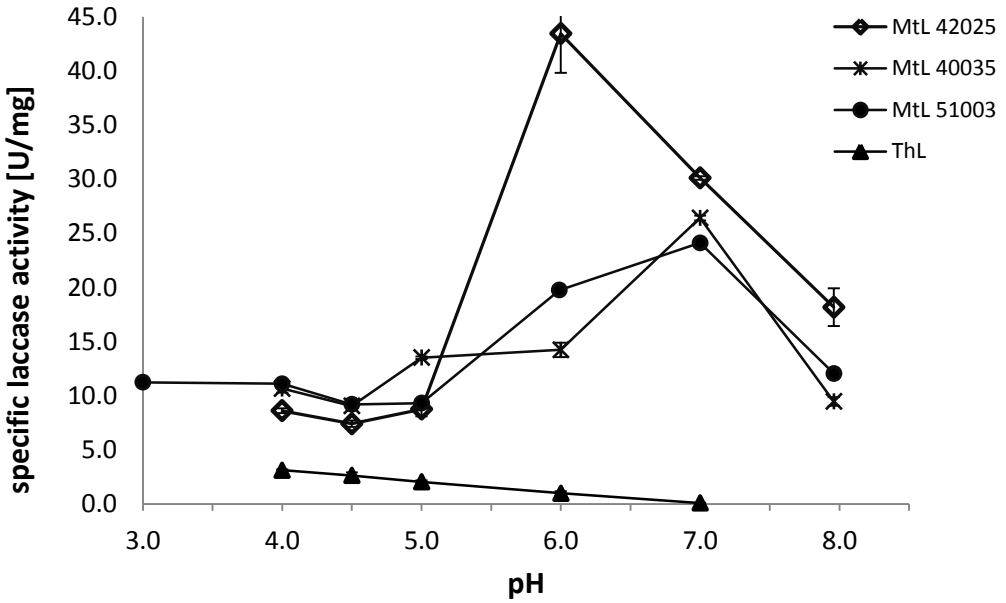

Figure 53: pH dependent specific activities [U/mg] of *Mt*L and *Th*L.

Screening for laccase mediators

Additional FTIR measurements of a long alkyd resin film with laccase and mediator were performed, varying the concentration of the mediators (ABTS, vanillin, acetovanillone) and the activity of *Mt*L.

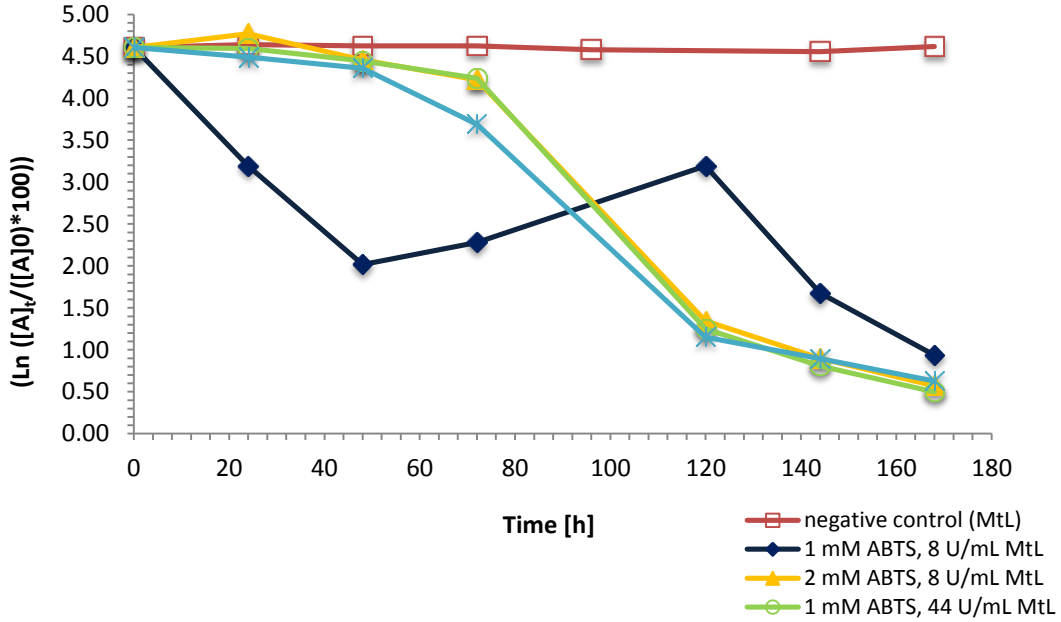

Figure 54: ATR-FTIR spectra of long alkyd resin showing the variation of cis-C=C-H symmetric stretch peak during the oxidation under the influence of various concentrations of ABTS and MtL. The ratio between the peak area at the time of the measurement and the area at the beginning was calculated for each mediator and the logarithmic plot of the data versus time is shown.

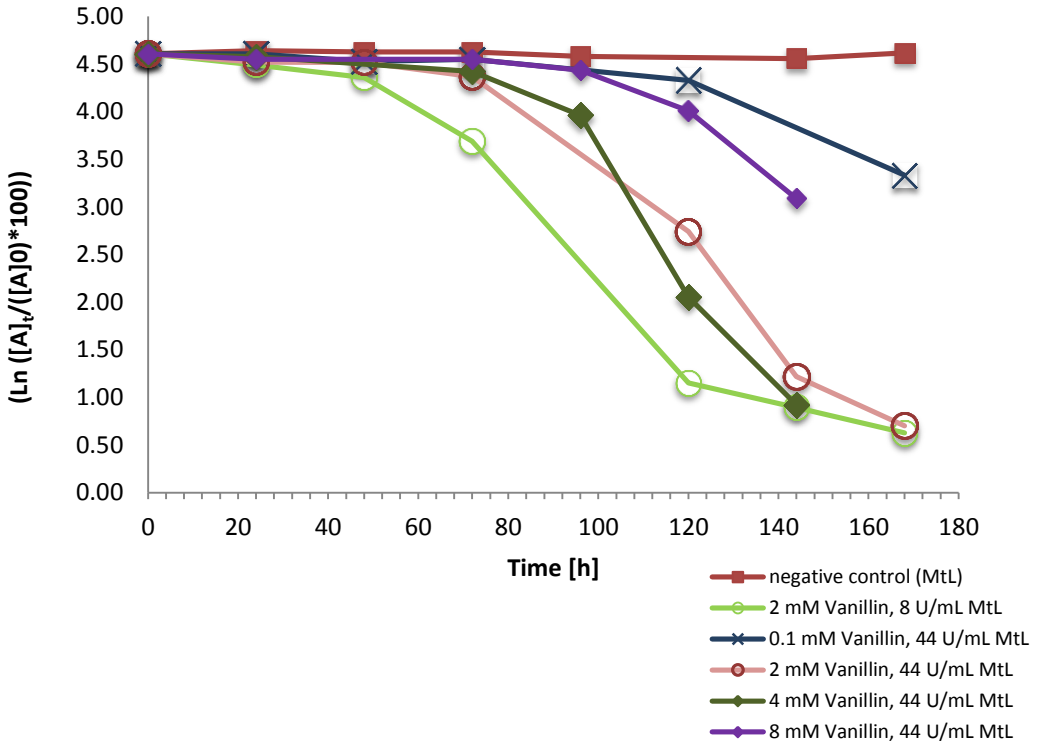

Figure 55: ATR-FTIR spectra of long alkyd resin showing the variation of cis-C=C-H symmetric stretch peak during the oxidation under the influence of various concentrations of vanillin and MtL. The ratio between the peak area at the time of the measurement and the area at the beginning was calculated for each mediator and the logarithmic plot of the data versus time is shown.

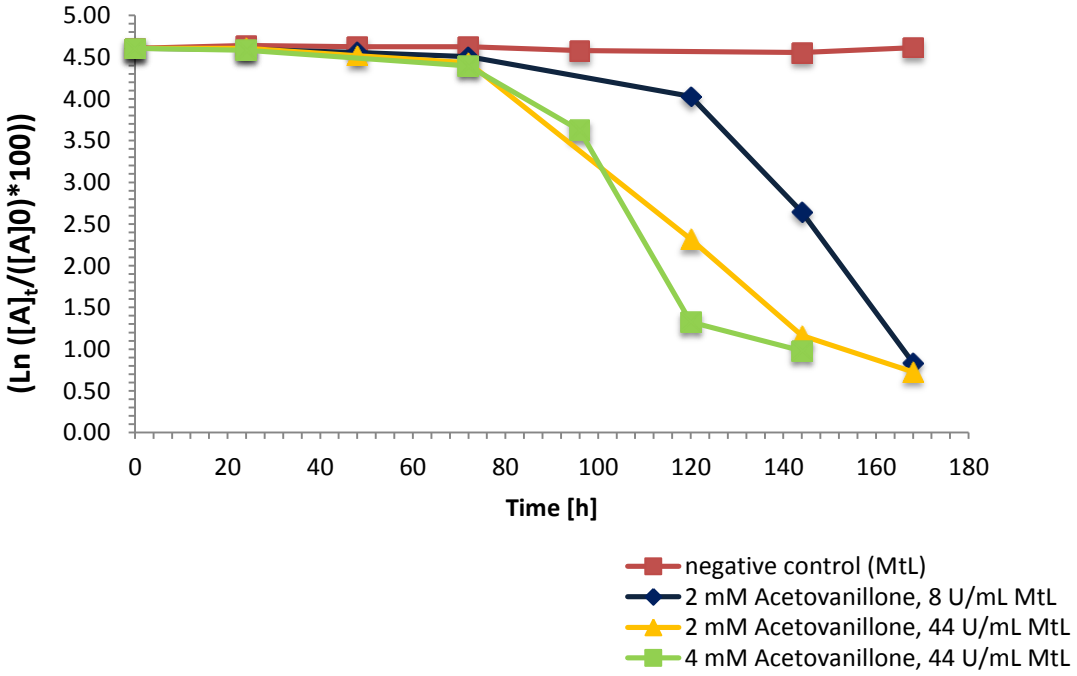

Figure 56: ATR-FTIR spectra of long alkyd resin showing the variation of cis-C=C-H symmetric stretch peak during the oxidation under the influence of various concentrations of acetovanillone and MtL. The ratio between the peak area at the time of the measurement and the area at the beginning was calculated for each mediator and the logarithmic plot of the data versus time is shown.

Process monitoring via Fourier transform infrared spectroscopy

Figure 57 illustrates a full range FTIR spectrum obtained from long alkyd resin after a reaction time of 24 h comparing the drying of an alkyd resin film, with and without mediator or laccase present.

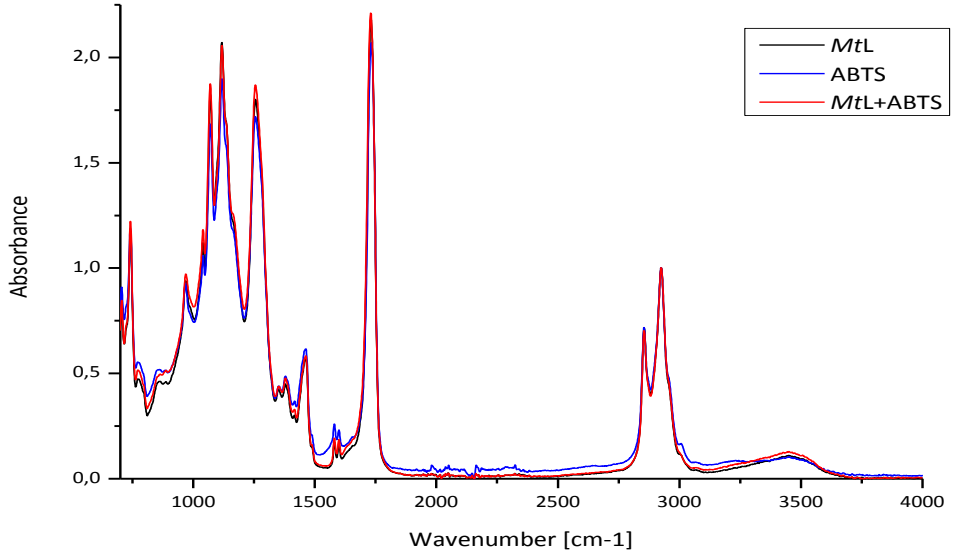

Figure 57: Normalized full range FTIR-spectrum of long alkyd resin, treated with mediator and laccase (red), laccase (black) or mediator (blue).

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